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A Recombinant *Bacillus anthracis* Strain Producing the *Clostridium perfringens* Ib Component Induces Protection against Iota Toxins

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The *Bacillus anthracis* toxinogenic Sterne strain is currently used as a live veterinary vaccine against anthrax. The capacity of a toxin-deficient derivative strain to produce a heterologous antigen by using the strong inducible promoter of the *B. anthracis pag* gene was investigated. The expression of the foreign gene *ibp*, encoding the Ib component of iota toxin from *Clostridium perfringens*, was analyzed. A *pag-ibp* fusion was introduced by allelic exchange into a toxin-deficient Sterne strain, thereby replacing the wild-type *pag* gene. This recombinant strain, called BAIB, was stable and secreted large quantities of Ib protein in induced culture conditions. Mice given injections of live BAIB spores developed an antibody response specific to the Ib protein. The *pag-ibp* fusion was therefore functional both in vitro and in vivo. Moreover, the immunized animals were protected against a challenge with *C. perfringens* iota toxin or with the homologous *Clostridium spiroforme* toxin. The protective immunity was mediated by neutralizing antibodies. In conclusion, *B. anthracis* is promising for the development of live veterinary vaccines.

Bacillus anthracis is a gram-positive spore-forming bacterium and is responsible for anthrax. It is an extracellular pathogen, and its virulence depends on the secretion of two exotoxins and the production of an antiphagocytic capsule encoded by the plasmids pXO1 (185 kbp) and pXO2 (95 kbp), respectively (14). Three bacterial secreted proteins, PA (protective antigen), LF (lethal factor), and EF (edema factor), encoded by the genes *pag*, *lef*, and *cya*, respectively, combine pairwise to form the lethal (PA + LF) and edema (PA + EF) toxins (14). These toxins are responsible for the major physiopathological effects observed during infection, i.e., edema and shock-like death (17).

Spores of the *B. anthracis* Sterne strain are used as a live vaccine against anthrax in cattle (9). This strain, attenuated by curing virulent bacteria of plasmid pXO2, still harbors pXO1 and develops in vivo, i.e., germinates, persists and/or multiplies, and produces antigens, including the anthrax toxins, that ultimately induce immunoprotection (11, 19, 34, 35). The PA component is essential for the protective response induced by the Sterne vaccine (19).

Live bacterial vaccines against brucellosis, *Salmonella* infections, cholera, or shigellosis have also been developed by attenuating the virulence of strains (4, 10, 20). Their capacity to induce protection is due to the synthesis of protective antigens which stimulate the immune system. In vivo delivery of heterologous antigens has been extensively investigated with bacteria, especially *Salmonella* strains, as live vectors (2, 8, 24, 31). Efficient stimulation of a serum antibody response depends on the amount of antigen produced in the host and its delivery outside the bacterial cell (7, 13, 24).

B. anthracis strains derived from the Sterne strain by defined deletions of a toxin gene(s) on pXO1 are potential live vectors (17–19). These strains, which are deficient in one or two toxin components, unlike strains cured of both plasmids pXO1 and pXO2, are able to induce an immune response (11, 19). This property is related to the development of spores in the host, since antibodies specific to vegetative bacilli are found in the sera of immunized animals. This phenomenon does not require the biological activity of anthrax toxins but appears to be dependent on other pXO1-encoded factors. In addition, the promoters of *B. anthracis* toxin genes seem appropriate for driving the synthesis of foreign antigens. In culture medium, transcription of *pag*, *lef*, and *cya* genes is strongly coactivated by specific environmental factors, such as bicarbonate and a temperature of 37°C, and is dependent on the positive regulator AtxA (1, 3, 12, 27, 33). In vivo, toxin synthesis appears to be controlled by similar mechanisms (3; unpublished results).

We investigated the production by *B. anthracis* of the Ib component of iota toxin from *Clostridium perfringens* type E (15, 30). This pathogen causes animal diseases, mainly calf and lamb enterotoxemias. The binary iota toxin, like anthrax toxins, is organized according to the A-B model. The Ib protein, the B component, binds to cells and mediates the intracellular delivery of Ia, the A component (21, 22, 29, 30); therefore, Ib is analogous to PA. Moreover, the amino acid sequences of Ib and PA share 54% similarity and 34% identity (15). In addition, Ib is a model antigen with potential veterinary applications since it induces an immunoprotective response against iota toxin (25). We therefore constructed a *B. anthracis* recombinant strain harboring a gene fusion between the *pag* gene promoter and the *ibp* gene, encoding Ib. The in vitro and in vivo expression of *ibp* and the protective immunity induced by the recombinant *B. anthracis* strain against iota toxins were analyzed.

MATERIALS AND METHODS

Bacterial strains and culture media. In this study, *B. anthracis* RP10 and RP31 were used (18). They were derived from the vaccinal Sterne strain 7702 (17) by deletion of the *lef* gene for RP10 and *lef* and *pag* genes for RP31. *B. anthracis* was generally grown at 37°C in brain heart infusion medium. To analyze the expres-

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sion of toxin genes, *B. anthracis* was grown at 37°C in R medium supplemented as appropriate with 0.4% (wt/vol) sodium bicarbonate (23, 27). *B. anthracis* spores were prepared as described previously (17). *Escherichia coli* TG1 was used as the host for DNA construction (26), and *E. coli* JM83(pRK24) was used as the donor in conjugation experiments (32). Antibiotics were used at the following concentrations: erythromycin, 5 μg ml^{-1}, for *B. anthracis*; kanamycin and spectinomycin, 40 and 60 μg ml^{-1}, respectively, for *E. coli* and *B. anthracis*. *C. perfringens* type E strain and *C. spiroforme* NCTC 11493 were used as sources of iota toxin components for challenge experiments (21, 22).

DNA techniques and plasmids. Methods for recombinant DNA isolation and manipulation were as described by Sambrook et al. (26). PCR was performed with primers specific for *pag*, *ibp* or *spc* genes: PAG1, GAGCTGCCCACCAA GCTAAACC; PAG2, CTTCTTTAAGCCCTTCAGTATCTTC; IBP1, CAAAT CACCATTTTTGATTGGCG; IBP2, GGAGATCAAAACCAACCTAAAACT; SPC3, CGCTGTTAATGCGTAAACCACC; SPC4, GGAGAGTGTGATGAT AAGTGGG.

Plasmid pBAFH115, used to construct the *ibp* expression plasmid, was derived from plasmid pBAFH113 (unpublished data) which itself was derived from the vector pAT113. The conjugative vector pAT113 is integrative in *B. anthracis* (6, 27) and harbors both an erythromycin resistance cassette and a kanamycin resistance cassette for selection (32). pBAFH115 carries (i) the *pag* regulatory region (nucleotides 1 to 1806), in which a *Nde*I site (CATATG) was introduced into the translation initiation codon; (ii) a single *Acc*65I restriction site; (iii) a spectinomycin resistance cassette, *spc* (6); and (iv) a 3′-end fragment of the *pag* gene (nucleotides 2871 to 4230) (36). A *Bam*HI-*Sph*I fragment corresponding to the *ibp* gene (nucleotides 2501 to 5743) from *C. perfringens* type E NCIB 10748 (15) was inserted into bacteriophage M13mp18 and used in the following constructs.

Construction of the *B. anthracis* recombinant strain. A *Nde*I restriction site was introduced into the translation initiation site of the *ibp* gene to allow a translational fusion with the *pag* regulatory region: the oligonucleotide-directed in vitro mutagenesis system version 2.1 (Amersham), the bacteriophage M13mp18 containing the *ibp* gene, and the oligonucleotide IBPNDE, TACATTTTTAATT TGTATATTCATATGTTTTCCTCC were used. The resulting phages were analyzed both by sequencing and by digestion with *Nde*I enzyme to identify mutants. A translational fusion between the *pag* regulatory region and the *ibp* structural gene was generated by inserting the *Nde*I-*Sph*I(blunt) fragment of *ibp* (from the translational initiation site to nucleotide 5743) into plasmid pBAFH115 cut with *Nde*I-*Acc*65I(blunt) (15). The resulting suicide plasmid, pBAIB113, was then transferred by mating from *E. coli* JM83(pRK24) into *B. anthracis* RP10 as described previously (27). *B. anthracis* transconjugants were selected for spectinomycin resistance and screened for erythromycin susceptibility. The *B. anthracis* clone BAIB resulted from integration of the construct from the suicide plasmid pBAIB113 by double crossovers into pXO1 at the *pag* locus. The wild-type copy of *pag* was therefore replaced by the *pag-ibp* fusion in BAIB, as verified by PCR with specific primers.

Purification of Ib produced by the BAIB strain. For large-scale production of the Ib component, the BAIB strain was grown for 16 h at 37°C under 5% CO_2 in 4 liters of R medium supplemented with sodium bicarbonate. The supernatant was collected and concentrated by ultrafiltration on Minitan (Millipore), and proteins were precipitated in 70% ammonium sulfate. About 15 mg of protein, composed mainly of the Ib 96-kDa form, was obtained. The precipitate was dialyzed against 10 mM Tris-HCl (pH 7.5) and loaded on a DEAE-Sepharose CL6-B column (Pharmacia). The column was washed with 100 mM NaCl in Tris buffer. The material was eluted with 200 mM NaCl in Tris buffer, dialyzed against 10 mM sodium citrate (pH 4.5), and chromatographed on a DEAE-Sephadex CL6-B column equilibrated with the citrate buffer. Proteins were eluted with a 0 to 100 mM NaCl gradient in citrate buffer. The major peak of protein corresponded to Ib. The fractions corresponding to Ib were pooled and treated with trypsin (200 μg ml^{-1}) for 30 min at 37°C, and soybean trypsin inhibitor (400 μg ml^{-1}) was added. This trypsin-treated protein preparation was used in the experiments.

Purification of Ia, Sa, and Sb components. The Ia protein (43 kDa) was purified from *C. perfringens*, and the Sa (45 kDa) and Sb chains were purified from *C. spiroforme* as described previously (21, 22). The Sb native protein (92 kDa) was proteolyzed with trypsin, as described for Ib, giving rise mainly to the 76-kDa active form.

Protein and immunoblot analysis. Proteins in culture supernatants were precipitated with 10% (vol/vol) trichloroacetic acid and were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). The gels were stained with Coomassie blue or subjected to immunoblot analysis with an Ib-specific rabbit polyclonal serum as primary antibody. Goat anti-rabbit immunoglobulin G (IgG) phosphatase alkaline conjugate (Sigma Immunochemicals) was used for immunodetection.

Toxicity assays, immunization, and challenge procedures. Adult, female, 6- to 10-week-old, pathogen-free Swiss OF1 mice were supplied by IFFA-CREDO (L'Arbresle, France) and were maintained in a protected environment. The biological activity of iota toxins was assayed by giving the animals intraperitoneal (i.p.) injections of equimolar amounts of Ia and Ib or of Sa and Sb in 0.5 ml of toxin buffer (phosphate-buffered saline [pH 6.3], 2% [wt/vol] gelatin). Mortality was monitored for 1 week. To determine the 50% lethal dose (LD_{50}), serial dilutions of iota toxin were injected into mice (four to five mice per group). By using the Probit method, the LD_{50} of *C. perfringens* iota toxin was estimated to be 1.2 μg of Ib and 0.6 μg of Ia and that of *C. spiroforme* toxin was 0.9 μg of Sb and 0.45 μg of Sa.

Animals (groups of 10 to 18) were immunized with strain BAIB on day 0 (single dose) or days 0 and 21 (two doses) by subcutaneous injection of 1×10^8 to 2×10^8 spores in 0.5 ml of saline (0.15 M NaCl). Two groups of control animals were given subcutaneous injections on day 0 with sterile saline or strain RP31. On day 35, all the animals were challenged with a lethal dose of *C. perfringens* iota toxin (3 μg of Ib and 1.5 μg of Ia) or of *C. spiroforme* iota toxin (2 μg of Sb and 1 μg of Sa), and survival was monitored for 1 week to estimate protection. The χ^2 test was used for statistical analysis.

Serological studies. Mice were bled from the retroorbital plexus on day 35 to obtain serum samples. Antibody titers (total mouse Ig) directed against vegetative extracellular antigens of *B. anthracis*, Ib, or Sb components were determined by an enzyme-linked immunosorbent assay (ELISA), as described previously (19). Antigens were prepared as described previously (19, 22) or as presented in Materials and Methods. Microplates were coated with 100 ng of the appropriate antigen per well and were incubated with various serum dilutions (1:100 to 1:204,800). Antibody binding was revealed with horseradish peroxidase-conjugated goat anti-mouse serum (1:1,000; Amersham) and 100 μl of an *o*-phenylenediamine (Abbott Laboratories) substrate solution. Finally, microplates were read at 492 nm, and antibody ELISA titers were defined as the serum dilution at which the absorbance at 492 nm is 0.5. Student's *t* test was used for statistical analysis.

Neutralization assays. Sera from BAIB-boosted mice or from naive mice were pooled and heated for 30 min at 56°C. For each mouse, 3 μg of Ib in 405 μl of toxin buffer was incubated for 1 h at 37°C with 45 μl of pure or diluted sera (the final dilutions of the sera ranged from 1:10 to 1:160). The mixture was then supplemented with 1.5 μg of Ia in 50 μl of toxin buffer and was injected into naive mice (3 to 5 animals per dilution) (5). Neutralization was performed similarly on *C. spiroforme* toxin with 2 μg of Sb and 1 μg of Sa. The neutralizing-antibody titer was scored as the highest dilution at which all animals survived.

RESULTS AND DISCUSSION

Construction of a *B. anthracis* strain producing Ib. Of the *pag*, *lef*, and *cya* genes, *pag* is the most strongly expressed, as assessed by *lacZ* transcriptional fusions and RNA analysis (3, 27). Therefore, the *pag* regulatory region was used for gene fusion to the *ibp* gene on a suicide plasmid containing a spectinomycin resistance cassette and the 3′ end of the *pag* gene (Fig. 1A). The resulting plasmid, pBAIB113, was transferred by mating into the LF-deficient *B. anthracis* RP10 (17). A *B. anthracis* transconjugant, called BAIB, resistant to spectinomycin was isolated and further studied. Strain BAIB had integrated the *pag-ibp* fusion by allelic exchange at the *pag* locus on pXO1 (Fig. 1A), thereby inactivating the *pag* gene. Since pXO1 is a natural resident plasmid, the construct was stable, and strain BAIB is therefore suitable for animal experiments. We verified that the BAIB strain, like the parental strain RP10, was avirulent for mice: its LD_{50} was $>10^9$ spores. Since BAIB is isogenic to the *B. anthracis* PA- and LF-deficient strain RP31 (18), we used RP31 as the control.

In vitro production of Ib by *B. anthracis* BAIB. The expression of the *pag-ibp* fusion by the *B. anthracis* recombinant BAIB was analyzed under various culture conditions. In the presence of bicarbonate, *pag*, *lef*, and *cya* transcription is increased 10- to 20-fold, leading to the production and secretion of anthrax toxins, which become the most abundant proteins in the culture supernatants (3, 14, 23, 27). Under uninduced conditions, the Coomassie blue-stained SDS-PAGE protein profiles of strains BAIB and RP31 were similar (Fig. 1B, lanes 5 and 7). In contrast, in presence of bicarbonate, an abundant protein with an apparent mass of 96 kDa appeared in BAIB supernatants (lane 4). Immunoblot analysis with Ib-specific antibody confirmed that this 96-kDa protein was Ib (Fig. 1C). Like anthrax toxin components, Ib was produced throughout the exponential growth phase (data not shown). These data indicate that expression of the *pag-ibp* fusion was indeed controlled by the *pag* promoter.

The Ib component was efficiently secreted by strain BAIB. Its signal sequence was therefore recognized by the *B. anthra-*

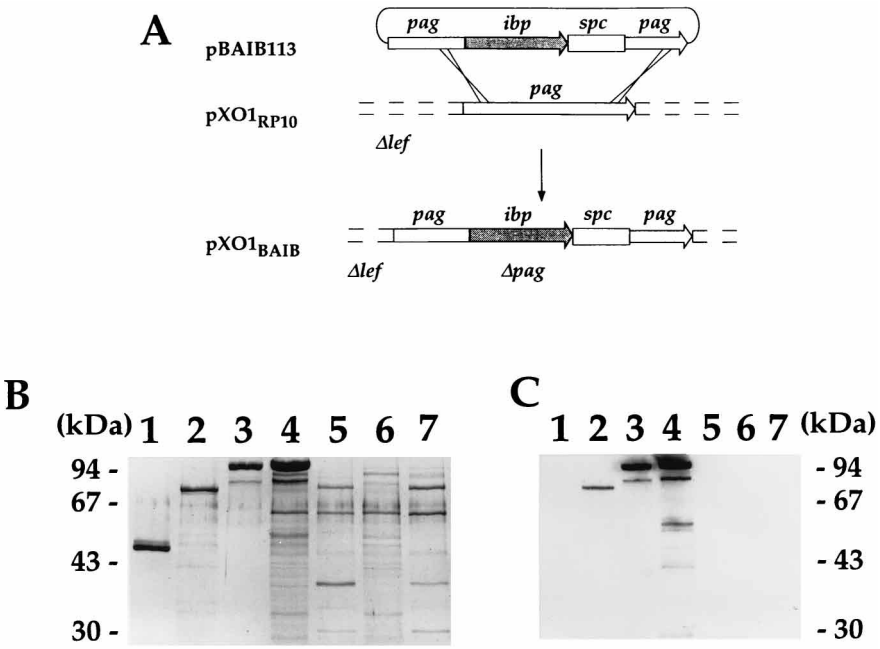

FIG. 1. Construction of the *B. anthracis* recombinant BAIB and analysis of in vitro production of Ib component. (A) Schematic diagram of the construction of *B. anthracis* BAIB. The *pag* upstream regulatory region was fused to the *ibp* gene. The fusion was linked to the spectinomycin resistance gene, *spc*, and the 3′ end of the *pag* gene. The construct on the suicide plasmid pBAIB113 was transferred to pXO1 in *B. anthracis* RP10 by homologous recombination between both the 5′ and 3′ ends of the *pag* gene. (B and C) Synthesis of Ib by strain BAIB. Supernatants (0.75 ml) of BAIB and of the control strain RP31 grown in R medium, in the presence or absence of sodium bicarbonate, were analyzed by SDS-PAGE and Coomassie blue staining (B) or immunoblotting with Ib-specific serum (C). Lanes: 1, Sa purified from *C. spiroforme* (0.5 μg); 2, trypsin-activated Sb from *C. spiroforme* (1 μg); 3, trypsin-treated Ib protein purified from strain BAIB (1 μg); 4 and 5, R-medium-plus-bicarbonate (lane 4) and R medium (lane 5) supernatant from strain BAIB; 6 and 7, R-medium-plus-bicarbonate (lane 6) and R medium (lane 7) supernatant from strain RP31.

cis secretion machinery. This is in agreement with previous findings for the production and secretion of another *Clostridium* protein, the carboxymethylcellulase from *Clostridium thermocellum*, by two *Bacillus* species, *Bacillus subtilis* and *Bacillus stearothermophilus* (28).

The Ib protein was purified from *B. anthracis* culture supernatant and was treated with trypsin as described for activation of components prepared from *C. perfringens* (Fig. 1B and C, lanes 3) (22, 30). The trypsin-treated Ib preparation contained the Ib active form (about 80 kDa) and, in the presence of Ia, provoked the expected toxic effects (21): cytotoxicity on Vero cells as observed by morphological alterations (data not shown), and lethality for mice. Thus, strain BAIB produced an Ib protein functionally similar to that of *C. perfringens*.

In vivo production of Ib by the recombinant *B. anthracis* BAIB. The in vivo properties of the *B. anthracis* recombinant BAIB were studied by using mice immunized once or twice with spores. Antibody responses in serum were analyzed by ELISA with the Ib protein and the homologous Sb component of *Clostridium spiroforme*. The iota toxins of *C. perfringens* (Ia plus Ib) and *C. spiriforme* (Sa plus Sb [Fig. 1B and C, lanes 1 and 2]) are very similar: (i) there is a cross-complementation between their respective components for biological activity, and (ii) hyperimmune sera raised against one toxin can neutralize lethal effects of the other and vice versa (29). Both *B. anthracis* BAIB and RP31 induced a significant antibody response against the vegetative extracellular antigens of *B. anthracis* (Table 1) (19). Therefore, BAIB, like the control isogenic strain RP31, is able to develop in mice, i.e., to germinate, to multiply, and to produce *B. anthracis* antigens in vivo. Strain BAIB elicited high levels of antibodies against Ib and Sb (Table 1), indicating that the *pag-ibp* fusion is functional in vivo. Antibody titers against Ib and Sb were 20-fold higher in animals which received two doses of BAIB spores than were those in animals which received only one dose. Therefore, the booster injection with the *B. anthracis* recombinant strain presumably induced an amplified secondary humoral response. These results confirm that the Ib component has common epitopes with Sb and is thus able to stimulate a cross-reactive immune response (16). However, in immune animals, the ELISA titers against Sb were lower than those obtained against Ib, indicating that the immunological cross-reaction between these two homologous molecules is only partial.

Our data suggest that the *pag* gene promoter can be used to drive strong expression of foreign genes in *B. anthracis* in the host. Interestingly, in a system in *Salmonella*, increasing the amount of heterologous antigen delivered in vivo stimulates immunity even in individuals which do not respond or which respond only weakly to low doses (7). *B. anthracis* therefore

TABLE 1. Humoral response induced by *B. anthracis* BAIB

Strain	Antibody titer[a] in serum in response to:		
	B. anthracis extracellular antigens	Ib	Sb
RP31	1,740	<100	<100
BAIB			
Single dose	800	8,490	900
Two doses	4,980	147,000	25,400

[a] Reciprocal geometric mean ELISA titers of serum Ig for 10 mice.

TABLE 2. Protection induced by *B. anthracis* BAIB against iota toxins

Strain	% Survival[a] after challenge with toxin from:		Serum neutralizing titer[b] against toxin from:	
	C. perfringens	*C. spiroforme*	*C. perfringens*	*C. spiroforme*
RP31	0	0	<1:10[c]	<1:10[c]
BAIB				
Single dose	60	72	ND[d]	ND
Two doses	100	100	1:40	1:10

[a] Protection was determined with groups of 10 to 18 animals after i.p. injection of 3 μg of Ib and 1.5 μg of Ia or 2 μg of Sb and 1 μg of Sa.

[b] Values represent the highest dilution of sera from immunized animals that protected 100% of naive mice against i.p. toxin challenge.

[c] Control sera from nonimmune mice were used. No protection was observed at a 1:10 dilution.

[d] ND, not done.

appears promising as a vehicle to elicit a humoral response in various hosts.

Protective immunity induced by *B. anthracis* BAIB. The protection induced by strain BAIB in mice was evaluated. Animals were challenged, 35 days after the first immunization, with a lethal dose of iota toxin from *C. perfringens* (LD_{50} ≈2.5) or *C. spiroforme* (LD_{50} ≈2). Immunization with *B. anthracis* BAIB spores but not with RP31 spores protected mice against the lethal effects. After challenge, 60 to 72% of animals which received one immunizing dose and all boosted animals survived (Table 2). The level of protection appears to follow the Ib- or Sb-specific antibody titers. The recombinant BAIB bacterium is thus a potential veterinary vaccine against enterotoxemia induced by iota toxin-associated infections (5).

To further correlate the protection with the humoral response induced by strain BAIB, the neutralizing activity of pooled sera from BAIB-boosted animals was compared to that from naive mice (Table 2). Nonimmune sera had no protective effect against the lethality induced by *C. perfringens* or *C. spiroforme* iota toxins in mice. In contrast, a 10- to 40-fold dilution of BAIB-immune sera neutralized 100% of the lethal effects. Therefore, the BAIB strain induces protection against iota toxins through production of Ib- or Sb-specific neutralizing antibodies. This immunity is similar to that developed by animals immunized with iota toxin(s) (5, 29). Finally, our results confirm that B-component-specific neutralizing antibodies are sufficient for immunoprotection against iota toxin (25).

In conclusion, *B. anthracis* is a potential live vector for veterinary vaccination. Toxin-deficient strains carrying stable genetic constructs on plasmid pXO1 can be obtained. In vivo, they are able to develop, to produce, and to adequately present foreign antigens. They thus induce a protective humoral response. In the future, multivalent vaccine strains could be designed to provide simultaneous protection against anthrax and other veterinary diseases.

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