A Study of Nonpathogenic *Francisella*, *Brucella*, and *Yersinia* Strains as Producers of Recombinant β-Endorphin

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Yersinia pseudotuberculosis, *Brucella abortus*, and *Francisella tularensis* strains producing a recombinant β-endorphin have been obtained. The highest production of this peptide, which displays physiological activity, was recorded for cells of the *Y. pseudotuberculosis* strain 2243 (pSK95E).

Key Words: *β-endorphin; producer strains; synthesis; biological activity*

In our previous study [2], in which the regulatory peptide β-endorphin additively synthesized by a microbial donor was evaluated for its influence on the physiological state of recipient organisms, we established that the psychoemotional status and nociceptive responses of CBA mice depend on the reproductive dynamics in these hosts of the *Francisella tularensis* strain generating a recombinant β-endorphin. The prolonged effect induced by this strain exceeded by 25-50% the mean index of pain sensitivity inhibition [15]. Native β-endorphin (8-10 μg/kg) elicited in mice only a short-term effect that varied from 0 to 200-300% [2].

In an effort to further boost the additive action of recombinant β-endorphin, we have now explored the possibility of producing a number of strains capable of more intensive and more prolonged synthesis of this peptide *in vitro* and in the vaccinated animal host.

With this aim in view, we selected known vaccine strains of *Brucella abortus* and *Francisella tularensis* as well as avirulent *Yersinia pseudotuberculosis* strains. These microorganisms are all capable of prolonged survival in the laboratory animals used in our studies [1,3,7].

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MATERIALS AND METHODS

In the study, we used *Escherichia coli* strains C600 and HB101 [6], *F. tularensis* strain 15 [2], *B. abortus* strain 19-BA [3], and *Y. pseudotuberculosis* strains 149 and 2243 [9]. *E. coli*, *Y. pseudotuberculosis*, and *B. abortus* cells were cultured in L-broth or L-agar [6]. *F. tularensis* cells were grown on erythrite agar with black albumin [5]. The β-endorphin gene within the pSK95E plasmid was kindly provided by O. I. Serpinskii and V. V. Kravchenko (from the Research Institute of Microbiology, Koltsovo, Novosibirsk Region, Russia). This plasmid was used to transform *E. coli* and *Y. pseudotuberculosis* cells as described previously [6]. *Y. pseudotuberculosis* cells, unlike those of *E. coli*, were cultured at 28°C. The β-endorphin gene was transferred to *B. abortus* cells by the conjugation technique [11]. For the transfers, the Sa plasmid was used as the vector [14]. All manipulations with DNA were carried out in accordance with the guidelines set out by Mazin *et al.* [6]. In addition, *F. tularensis* 15 cells inheriting the β-endorphin gene in the chromosome [2] were used for analysis.

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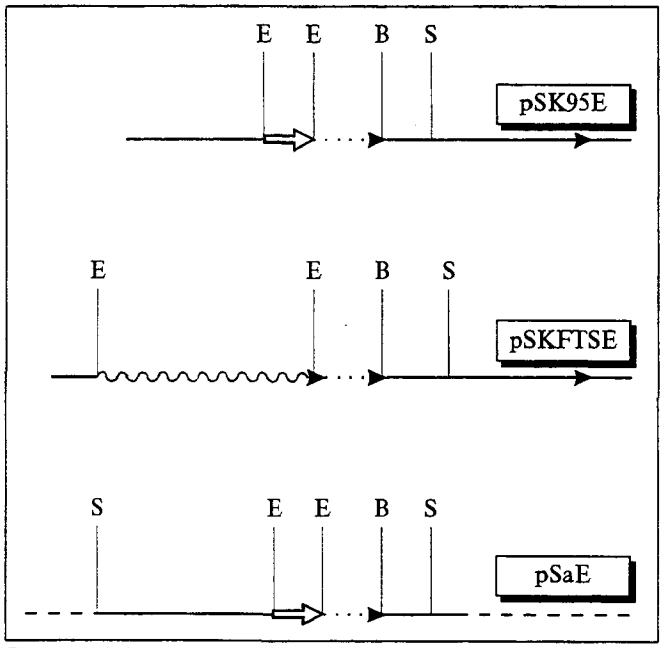

Fig. 1. Schematics of the genetic constructs providing for β-endorphin gene expression in *E. coli* (pSK95E), *Y. pseudotuberculosis* (pSK95E), *F. tularensis* (pSKFT5E), and *B. abortus* (pSaE) cells. The broad open arrows denote the lac UV5 promoter; the broken arrows with dots, the β-endorphin gene; the continuous arrows, the tet gene of the pBR322 plasmid; the continuous line, the sequence of the pBR322 plasmid; the wavy arrow, a fragment of the *F. tularensis* chromosomal DNA; and the broken line, the SA plasmid sequence. Restriction endonucleases are designated by symbols as follows: *E*: EcoRI, *B*: BamHI, *S*: SalI.

β-Endorphins synthesized by producer cells were assayed radioimmunologically using a standard reagent kit (INC, USA). As samples, cell lysates of each of the producers were used in a dose of 2×10^{10} CFU (colony-forming units). The lysates were prepared by treating cells with 10 mM KNa-phosphate buffer containing 0.9% NaCl and 2 mg/ml lysozyme. To lower proteolytic activity of the lysates against the recombinant β-endorphin, lysis was carried out at 4°C for 30 min. Additional disintegration of the cells was accomplished through freezing-thawing in cycles (-70°C - room temperature). The thawed-out samples were centrifuged and the supernatants, diluted if necessary 10-fold in 1% BSA (bovine serum albumin)-borate buffer, were used for further analysis. Samples with a known β-endorphin concentration were employed as standards.

Opioid activity of the recombinant β-endorphins relative to the native peptide was determined as described elsewhere [10] using isolated smooth-muscle preparations of guinea pig ileum. Test samples were prepared from lysates used in the radioimmunoassay by double extraction with acetone followed by evaporation in a rotor evaporator and resuspension in Krebs-Henseleit's solution [12]. The prepared samples were added directly to a bath for isolated organs. Lysates of cells that did not inherit the β-endorphin gene served as negative controls. As positive controls, β-endorphin (Serva) in a concentration of 0.3 μg/ml and morphine (Serva) in a concentration of 0.6 μg/ml were used. Ileum contractions were recorded using an isotonic transducer (HSE). Specificity of the test preparations' activity was assessed by blockage of their effects on ileum contractility by naloxone (Endo Laboratories), a specific antagonist of opioid peptides (2 mg/ml).

The stability of β-endorphin gene inheritance by *Y. pseudotuberculosis* 2243 (pSK95E) cells was determined by evaluating the β-endorphin-producing capacity of a culture isolated from organs of rats infected with that strain. For this, 20 white rats were each injected with 1 ml of physiological saline containing a culture of the test strain, after which two rats were decapitated each day for a total of 10 days and cultures from their liver, lung, and spleen were seeded on L-agar. If cultures were consistently recovered, they were assayed for β-endorphin as indicated above, and the percentage of those cells in a cell population that had retained tetracycline resistance was also determined.

RESULTS

The schemes of genetic constructs used in this study for obtaining a β-endorphin-producing strain are shown in Fig. 1. The pSK95E plasmid used for transforming *E. coli* and *Y. pseudotuberculosis* cells contained the β-endorphin gene together with the tet gene of the pBR322 plasmid in one reading frame. Processing of the peptide was accomplished using the translation terminator TAG which separates these genes. The expression of both genes was controlled by the lac UV5 promoter inserted at EcoRI sites directly before the initiating ATG codon of the β-endorphin gene. To transform *F. tularensis* cells, the pSKFT5E plasmid was used, in which a fragment of the *F. tularensis* chromosomal DNA served as the promoter [8]. Transformants of *E. coli*, *Y. pseudotuberculosis*, and *F. tularensis* cells were selected on the basis of tetracycline resistance of their clones, as the location of the β-endorphin and tet genes in the same operon with a single promoter and single terminator indicated that these genes should be expressed simultaneously in the microorganisms under study.

The β-endorphin gene was introduced into *B. abortus* cells by conjugation within the pSaE plasmid in which the pSK95E plasmid had been incorporated into the single restriction site SalI of

the Sa plasmid that did not affect the main functional regions of this plasmid [14]. In the conjugation, donors were strain C600 cells containing the pSaE plasmid, while recipients were strain 19-BA cells resistant to nalidixic acid. *Brucella* transconjugates inheriting the pSaE plasmid in an extrachromosomal state were selected according to the resistance of the clones to nalidixic acid and to kanamycin, the gene for resistance to the latter being encoded on the SA plasmid [11].

Work to identify variants of the organisms under study with appropriate phenotypic characteristics resulted in the selection of 10 clones of each variant. The level of β-endorphin expression was then determined for each of the selected clones. The results of determining β-endorphin gene expression in various hosts are shown in Table 1. The lowest amounts of the peptide were recorded in lysates of *B. abortus* cells inheriting the pSaE plasmid. β-Endorphin gene expression in these cells was almost five times lower than in *E. coli* cells inheriting the same plasmid, although the two strains showed virtually the same kanamycin resistance (400 μg/ml). A similar effect was observed when comparing expressions of the kanamycin and tetracycline resistance genes of the pTH10 plasmid inherited by *E. coli* C600 and *B. abortus* S19 cells. The levels of kanamycin resistance were the same (500 μg/ml), whereas the minimal inhibitory tetracycline concentrations were 125 μg/ml for *E. coli* and only 25 μg/ml for *B. abortus* [11]. This fact was probably associated with the presence of promoter specificity in *Brucella* cells, so that the use of the lac UV5 promoter for β-endorphin production in 19-BA cells did not allow the same level of β-endorphin expression to be attained as in *E. coli* cells, for which this promoter is homologous.

A low level of expression was also recorded in *F. tularensis* cells, even though a homologous promoter was used to effect β-endorphin synthesis. The weak β-endorphin production by *F. tularensis* cells was probably associated with the use of an integrative vector constructed on the basis of an extended chromosomal DNA fragment from this organism, for it has been established that allelic inclusion of a gene in a homologous sequence usually leads to low copying of the included gene - the β-endorphin gene in this case [13]. In *E. coli* cells, the use of this same vector, inherited in an extrachromosomal state, resulted in a level of β-endorphin production that exceeded fourfold that in *F. tularensis* cells.

Substantially higher levels of β-endorphin gene expression were recorded for *Y. pseudotuberculosis*

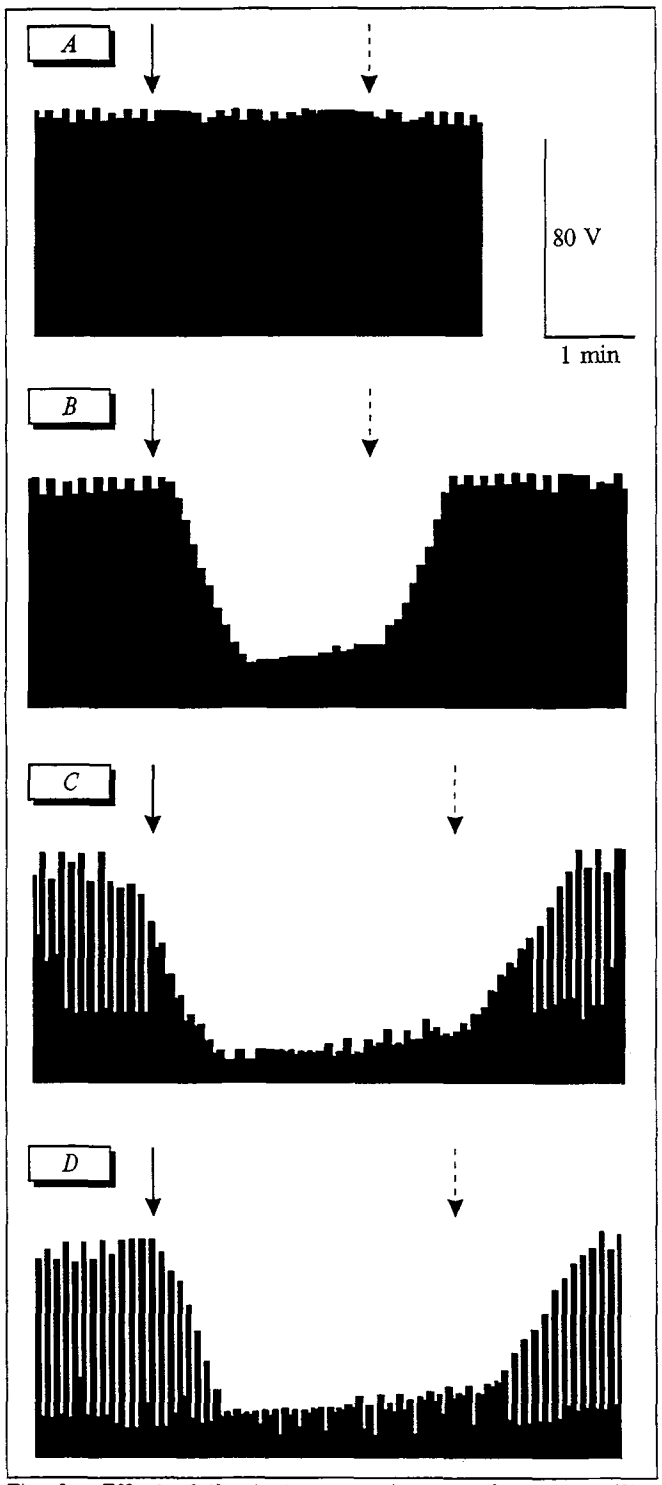

Fig. 2. Effect of the test preparations on the contractility of guinea pig ileum. Solid arrows: time when the preparation was added; broken arrows: time of naloxone introduction. *A*) *Y. pseudotuberculosis* 2243 cell lysate; *B*) morphine; *C*) commercial β-endorphin; *D*) 2243 (pSK95E) cell lysate.

cells. The pSK95E plasmid, which makes possible β-endorphin synthesis in these cells, had been constructed previously to create a β-endorphin producer on the basis of *E. coli* and appears to fit well to the genetic apparatus of *Y. pseudotubercu-*

TABLE 1. Levels of β-Endorphin Gene Expression in *E. coli*, *Y. pseudotuberculosis*, *B. abortus*, and *F. tularensis* Cells

Strain	b-Endorphin production, pmol/liter
E. coli C600 (pSK95E)	126 (111-141)
Same (pSaE)	84 (72-96)
Same (pSKFT5E)	85 (70-100)
Y. pseudotuberculosis 149 (pSK95E)	95 (78-112)
Y. pseudotuberculosis 2243 (pSK95E)	120 (108-132)
B. abortus 19-BA (pSaE)	18.7 (17.1-21.3)
F. tularensis 15 (pSKFT5E)	21 (14.1-27.9)

losis cells which, like *E. coli* cells, are members of the family Enterobacteriaceae. The highest level of expression recorded in strain 2243 (pSK95E) cells corresponded to the level of β-endorphin production by C600 (pSK95E) cells and was six times higher than that observed for *B. abortus* and *F. tularensis* cells. The 2243 (pSK95E) strain was therefore selected for further study, which involved determining the physiological activity of the β-endorphin synthesized by microbial cells and evaluating the stability of β-endorphin gene inheritance by cells of this strain during their persistence in the rat host.

Physiological activity of the peptide was evaluated by comparing the specificities with which the preparations under study bound μ-receptors of guinea pig ileum. It can be seen in Fig. 2 that, unlike the strain 2243 cell lysate, which virtually failed to affect ileum contractility (Fig. 2, *A*), the lysate of 2243 (pSK95E) cells caused inhibition of longitudinal smooth muscle contraction similar to the inhibition produced by opiates (Fig. 2, *B* and *C*). Specificity of the action of the test preparations was confirmed by the use of naloxone - an opiate antagonist with a higher affinity for μ-receptors of guinea pig ileum [4]. Naloxone abolished the effect of the test preparations and restored the oscillations to a pattern observed before these preparations began to act on m-receptors (Fig. 2, *B-D*). These findings indicate that the β-endorphin synthesized by *Y. pseudotuberculosis* cells displays marked physiological activity.

When strain 2243 (pSK95E) cultures were isolated from rat organs, the most intensive growth was shown by the cultures from the spleen. Moreover, the cultures persisted in the spleen longer (6 days) than in the lung (5 days) or the liver (4 days). On days 7-10 postinfection, no culture could be recovered from any of the organs. It should be noted that, regardless of the time of recovery, each of the cultures was still able to synthesize β-endorphin, even though the proportion of tetracycline-resistant clones in the cell population recovered from the spleen had fallen to 44% by day 6 of persistence in the host.

To summarize, the production of recombinant β-endorphin was highest in *Y. pseudotuberculosis* cells, in which the level of expression exceeded six times that recorded for *B. abortus* and *F. tularensis* cells. The peptide synthesized by *Y. pseudotuberculosis* cells displayed marked physiological activity and strain 2243 (pSK95E) cells persisted up to 6 days in rat organs and retained their capacity for β-endorphin synthesis regardless of the time when the culture was recovered. The *Y. pseudotuberculosis* strain 2243 (pSK95E) was therefore selected for further experiments to study the influence of the additively synthesized peptide on the physiological state of organisms that carry this strain.

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