MICROBIOLOGY AND IMMUNOLOGY

Stability of Avirulent Phenotype and Capacity of Limited Persistence of *Salmonella enteritidis* Avirulent Mutant in Mice

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S. enteritidis E-23 mutant possessing cya-mutation suppressive for CRP phenotype was obtained from virulent *S. enteritidis* 1791. The mutant has a stable avirulent phenotype and is persistent in mouse spleen for 45 days after oral administration.

Key Words: *Salmonella enteritidis; cAMP; mutant; stable avirulent phenotype*

The development of a new-generation vaccines requiring a lesser number of immunizations, administered via natural routes, and creating immunity to several infections is a pressing task. Among such vaccines can be gene-engineered vaccines based on attenuated vector strains, which can produce protective antigens of viral, bacterial, and parasitic origin [7,9,12]. Deciphering of molecular mechanisms of the pathogenesis of salmonellosis permits regarding *Salmonellae* as the optimal vectors carrying heterogeneous antigens to the immune system [7-9]. After oral administration, *Salmonellae* penetrate the intestinal epithelial barrier [8], mainly via M-cells [11], and multiply both in local intestinal lymphoid formations and in the parenteral lymphoid tissues (liver, spleen, and lymph nodes) [7-9]. Consequently, they can transport heterogeneous antigens from the intestine to lymphoid tissue. Oral administration of *Salmonellae* as vectors of heterogeneous antigens results in the formation of local and total-system humoral and cell-mediated immune response to *Salmonellae* and to heterogeneous antigens transmitted by them [10].

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According to the WHO requirements, a strain transmitting heterogeneous antigens should possess a stable avirulent phenotype and carry no markers of antibiotic resistance [14].

Among various types of avirulent *Salmonella* mutants, mutants deficient for the cAMP-regulatory system have been recognized as vector strains: the so-called cya- and crp-mutants [3,9]. In Russia, the main etiologic agent of salmonellosis is *S. enteritidis;* therefore, our purpose was to study the stability of avirulent phenotype of its mutant with the CRP phenotype and the capacity of this mutant to persist in mice after oral administration.

MATERIALS AND METHODS

The following strains were used: wild type *S. enteritidis* 1791, *S. enteritidis* avirulent mutant E-23, *S. typhimurium* B-2/1: cya, crp (2), *S. typhimurium* LT-2: met E. Transduction with P22 phagolysate and assessment of the kinetics of *Salmonella* multiplication in liquid minimum medium A [5] were carried out routinely [4]. LD_{50} and kinetics of *Salmonella* multiplication in mouse spleen were determined in outbred albino mice weighing 12-14 g as described previously [1]. The animals were sacrificed under ether narcosis.

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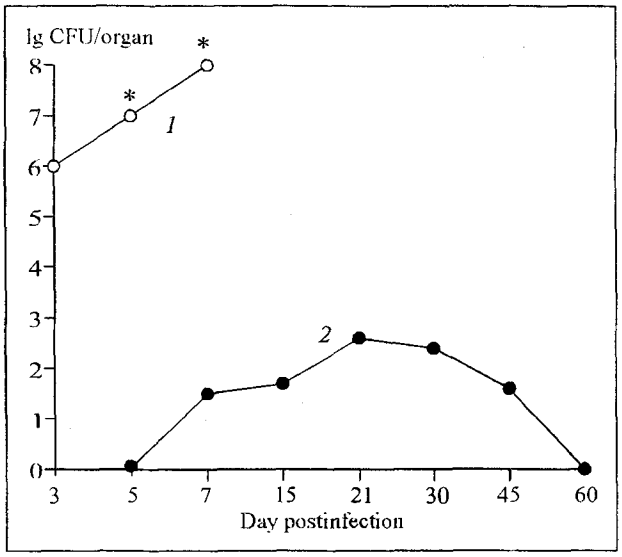

Fig. 1. Persistence of *S. enteritidis* 1791 and *S. enteritidis* E-23 in mouse spleen after oral infection in a dose of 10^7 CFU. *1*) mice infected with wild type *S. enteritidis* 1791; 2) mice infected with *S. enteritidis* E-23 mutant. *Inoculations from dead mice organs.

Salmonellae were counted in the spleen and liquid minimum nutrient medium after inoculation in McConkey's agar (Sigma). The results were expressed as the number of colony-forming units (CFU). 3':5'-Cyclic-AMP (Sigma) and methionine, cysteine, isoleucine, and valine (Reanal) were used.

RESULTS

S. enteritidis E-23 mutant was derived from *S. enteritidis* 1791 strain by transduction after treatment of the initial strain with P22 phagolysate multiplied on *S. typhimurium* B-2/1 mutant: cya, crp. *S. enteritidis* E-23 mutant was selected for the citrate negative marker, because of the lack of capacity to utilize citrate is a phenotypic manifestation of cya- and crp-mutations of *Salmonellae* [3,6]. *S. enteritidis* E-23 were isolated as a strain incapable of utilizing citrate even in the presence of exogenous cAMP in concentrations 1-5 mM in the medium. Study of the kinetics of the mutant multiplication in liquid glucose-minimal nutrient medium A showed a lag-phase of 105 min, whereas for cya- and crp-mutants of other Salmonella species this value was no more than 60 min [3]. Auxotrophicity markers to isoleucine, valine, methionine, and cysteine are cotransduced with the cya- and crp-genes [13], and therefore, we studied the effects of these amino acids on the kinetics of the mutant multiplication in liquid glucose-minimal medium A. The addition of methionine to the medium has cut the lag-phase to 60 min. The other parameters of multiplication kinetics did not depend on the presence of methionine in the medium, and after a 6-hour culturing, the bacterial harvest in medium with methionine was $2.8\pm0.15\times10^8$ CFU/ml and in starvation medium $2.0\pm0.33\times10^8$ CFU/ml. Other tested amino acids did not affect the lag-phase. Therefore, phagolysate P22 was obtained on *S. enteritidis* E-23 mutant, with which transduction was carried out using *S. typhimurium* LT-2 recipient strain: met E. During transduction the recipient strain was rendered the capacity to grow in the absence of methionine and (with 5% frequency) the phenotypical CYA sign, capacity to utilize citrate only in the presence of exogenous cAMP. Thus, we suggested that *S. enteritidis* E-23 possesses cya-mutation suppressed for the CRP phenotype.

The virulence of *S. enteritidis* E-23 mutant was decreased in comparison with the parental strain: lg LD_{50} of wild type strain was <1 upon intraperitoneal infection of mice *vs.* 8.5 in the mutant. The stability of avirulent mutant phenotype was studied as follows. Mice were intraperitoneally infected with *S. enteritidis* E-23 in a dose of 10^4 CFU, on day 10 the mice were sacrificed, and the spleen was removed, from which *S. enteritidis* E-23 was isolated. A suspension was prepared from the bacterial culture isolated from the spleen, and the next group of mice was infected with this suspension. The strain was passaged 8 times on mice. After the last passage, the LD_{50} of *S. enteritidis* E-23 isolated from mouse spleen was 9, i.e., *S. enteritidis* E-23 strain retained a stable avirulent phenotype. The vector of heterogeneous antigens must persist in the lymphoid tissue after oral administration, therefore, we tested the capacity of *S. enteritidis* E-23 to persist in mouse spleen after oral administration. Mice were orally infected with *S. enteritidis* E-23 and wild type *S. enteritidis* 1791 strain in a dose of 10^7 CFU. *Salmonellae* were counted in the spleen on days 3, 5, 7, 15, 21,30, 45, and 60 postinfection. The harvest of wild type strain isolated from the spleen was 10^6 CFU as early as 2 days postinfection (Fig. 1). Mice died in 7 days. The amount of the initial strain isolated from dead mice spleens was about 10^8 CFU.

S. enteritidis E-23 mutant administered to mice in the same dose did not cause the death of animals. It was first isolated from the spleen on day 7 postinfection and persisted in this organ for 15 days at the level of 10 CFU. By day 21, the number of *Salmonellae* increased to $3.89\pm0.92\times10^2$ CFU and persisted at the level of 100 CFU for up to 30 days. By day 45, the number of *Salmonellae* in the spleen decreased 10-fold and became 40 ± 0.6 CFU, on day 60 the microorganism was no longer isolated from the organ.

Thus, *S. enteritidis* E-23 mutant with cya-mutation suppressed for CRP phenotype has a stable

avirulent phenotype and is capable of limited multiplication in parenteral lymphoid tissues after oral administration.

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Immunohistochemical Localization of Somatotropin Receptors in Rat Liver Cells. Effect of Sex and Hormonal Status

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Peculiarities of cell and tissue distribution of somatotropin receptors in liver cells of male rats and intact, pregnant, and estrogenized female rats is studied using an indirect immunohistochemical method. Experiments reveal equal and positively regulated by female hormones expression of somatotropin receptors in all hepatocytes.

Key Words: *somatotropin receptors; rat liver; immunohistochemical analysis; sex-dependent expression*

Somatotropic hormone receptors (STHR) in rat liver are translated from at least two mRNA types with different 5'-flanking regions, arising due to initiation of transcription from two different promoters of the STHR gene [9]. A question arises of whether these processes can proceed simultaneously on one cell. One approach to solving this problem is an immunohistochemical analysis of STHR distribution in liver cells of animals with different levels of the STHR type I mRNA expression (intact males and females, and pregnant and estrogenized females [9]). This analysis is of interest in view of a comparison of liver cell sensitivity to STH and expression of STH-dependent proteins.

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MATERIALS AND METHODS

Experiments were carried out on 14 mature albino rats weighing 200-250 g (2 normal males, 5 normal and 2 pregnant (gestation days 15-18) females, and 5 females intramuscularly injected with estradiol in propylene glycol (15 μg/0.5 ml once a day for 10 days). The animals were decapitated, and the liver was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 20 min at 4°C. Sections (3-μ thick) of liver tissue were embedded in Paraplast,

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