Biosafety investigations in an r-DNA production plant

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Abstract. Employees of a biotechnological production plant for recombinant interferon-α-2a have been participating in a 1-year study on occupational hygiene and the resulting biosafety aspects. Most of the employees have been employed in the plant for more than 6 years. Weekly stool samples were analysed for tetracycline (used as selection marker)-resistant coliforms as well as for rDNA (IFN gene) (interferon gene) and for the production organism. Various analytical methods, including the polymerase chain reaction, were applied to show that neither rDNA nor the production organism could be found in any of the stool samples and that there was no change or trend in the gut flora with respect to tetracycline resistance. In addition it could be shown that the tetracycline-resistance gene, as well as the rDNA, are completely inactivated in the course of the production process and thus no further recombination can take place. Blood samples were taken to show that none of the employees had anti-product antibodies.

Introduction

In the application of recombinant DNA technology to bacteria such as Escherichia coli, one or two selection markers have generally been used to retain the vector (including inserts). Among these markers are often antibiotics, which are applied in human therapy, thus representing a potential risk for the spread of resistant bacteria or genes into the environment (Hummel et al. 1986; Levy 1987). In large-scale production of recombinant proteins, optimum occupational hygiene in the handling of antibiotic-resistant rDNA microorganisms or the antibiotics themselves, combined with good manufacturing practice (GMP) are therefore of the utmost importance in avoiding the spread of resistant genes in staff or the environment.

Experiments on the viability of E. coli K12 in the human intestine of volunteers have shown that these ‘down-mutated’ E. coli K12 are not able to survive or colonize the gut for more than 7 days after ingestion (Anderson 1975). Insufficient occupational hygiene in rDNA production could lead to the eventual ingestion of production organisms or antibiotics and thus to the spread of resistant genes. Since there are only limited data available as yet (Petrocheilou and Richmond 1977), we present some experimental data from studies that we have carried out during the last 10 years of large-scale production. This report summarizes a study, carried out over a period of 12 months, on occupational hygiene of personnel working in the biotechnological production plant for recombinant interferon-α-2a using E. coli. The process is operated in biosafety class 1 (Frommer et al. 1989) or GILSP (Good Industrial Large Scale Practice) according to the OECD guidelines (OECD 1986) and according to the EC (1990) council directive 90/219/EEC.

The main questions to be answered by these investigations were:
1. Can we observe changes in the antibiotic resistance of the gut flora of the staff?
2. Can we find the production organism or rDNA in the gut flora of the personnel?
3. Can we efficiently destroy the rDNA and resistance genes in the process?
4. Can we find anti-product antibodies in the serum of the employees?

Materials and methods

Stool sampling. The thiamine-auxotrophic strain E. coli K12/294 (ATCC 31446) was used in all investigations. It was transformed with a vector derived from pBR322 (Goeddel et al. 1980), containing a tetracycline-resistance gene and constitutively working trp promoters. For investigations of the gut flora the personnel of the production plant (five men) and the in-process control laboratory (two women) were involved. Except for two persons, all employees had been involved in this work for more than 8 years. All staff members have been permanently under medical supervision.

Stool samples were collected each week (except for holidays, etc.) at the same time from every person for 50 weeks and proc-
cessed within 8 h by a specialized company (Medibact, Basel, Switzerland). Together with the stool samples, a protocol giving detailed information on important events, such as holiday, illness and therapeutic treatments was completed. The stool samples were screened for tetracycline-resistant coliforms. For this purpose, 1 g fresh stool was thoroughly suspended in 10 ml sterile saline. One drop thereof was used to spread on MacConkey agar (Difco 1984) plates, containing 5 mg/l of tetracycline. After incubation at 37°C for 16 h, a maximum of ten colonies, differing in morphology from each other, were picked and 'preidentified' by the biochemical characterisation system of Enterotube II (Hofmann-La Roche 1981). Colonies, showing an Enterotube code different from the previous isolates were used for further identification, the majority of the rest being discarded. For the validation of the isolation sensitivity for stool organisms, varying cell numbers of the production strain were mixed into stool samples as follows: (a) no addition of cells of the production strain as reference; (b) addition of 3.6 × 10⁶ cells/g; (c) addition of 10⁷ cells/g; (d) addition of 10⁸ cells/g stool sample.

All coliforms isolated during the 1-year study were characterized by various specific tests and compared to the rIFNα-2a production strain.

**Host specific identification tests.** Eosin Methylene Blue (Difco 1984), differentiation agar was used for the detection of Gram-negative enteric bacteria, such as E. coli.

The thiamine requirement was tested on a minimal agar medium containing 5 mg/l of thiamine-HCl. No growth was allowed for auxotrophic strains without vitamin B₆.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) of the soluble cell extracts was carried out to compare the cell proteins of the production strain with the stool isolates.

**Vector-specific identification tests.** Tetracycline resistance was tested by adding 5 mg/l of the antibiotic to L-broth agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 20 g agar, ad 1000 ml H₂O).

The recombinant DNA product [rIFNα-2a] was quantitatively analysed after extraction of the cells with 7 M GuHCl (Guanidine Hydrochloride). The extract was submitted to an enzyme-linked immunosorbent assay (ELISA) sandwich test using Li-9 and Li-1 antibodies (Staehelin et al. 1981). The cut-off limit was as low as 1 ng/ml DNA of the production strain was used as reference template.

**Polymerase chain reaction (PCR) tests for rDNA (coding for rIFNα-2a) and for the tetracycline-resistance gene** were carried out using four primers of each coding sequence. The extracted plasmid DNA of the production strain was used as reference template.

**Anti-interferon antibodies.** The serum of 15 employees (fermentation, downstream processing, in-process control laboratory personnel) were analysed for anti-interferon antibodies in a status investigation in the course of this study. For this purpose blood samples were taken and analysed in a double sandwich ELISA test (Hennes et al. 1987). The cut-off limit was 50 interferon-binding units.

**Production process.** Refer to the flow diagram for the production process for rIFNα-2a bulk material. At the end of the production fermentation process all Escherichia coli cells are inactivated and the DNA is disintegrated by H₂SO₄.