Delayed hypersensitivity in the rabbit after injection of viable 'NSU *Corynebacteria*'

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'NSU *Corynebacteria*' are commensals of the normal male and female urogenital tracts (Furness, Kamat, Kaminski, and Seebode, 1973). They are so called because they were first described in association with NSU (Furness and Csonka, 1966), and the term is retained so that they should not be confused with other diphtheroids, which in contrast are easily isolated on commercial media and are well-known commensals.

Although commonly commensals, 'NSU *Corynebacteria*' have been isolated in pure culture from the epididymis of patients with acute epididymitis (Furness and others, 1971a, 1974) and from the bulbous urethra of patients with non-specific urethritis (NSU) (Furness and Csonka, 1966; Furness and others, 1971b). These reports suggest that some strains may cause disease while others are saprophytes. However, some of the conceivably pathogenic strains isolated from the epididymis of patients with epididymitis and the bulbous urethra of patients with NSU show characteristic biochemical reactions which differentiate them from the commensal 'NSU *Corynebacteria*' (Furness and others, 1973). Since Reiter's disease and chronic NSU have manifestations suggestive of an allergic syndrome, the ability of 'NSU *Corynebacteria*' to induce hypersensitivity and to produce antibodies in rabbits has been investigated, and the results are reported below.

Material and methods

Rabbits were injected with six strains of viable 'NSU *Corynebacteria*' (numbers 171EaC₁, 174EaC₁, 196EaC₃, 197EaC₃, 204C, 208Q) isolated from the epididymis of patients with acute epididymitis (Furness and others, 1971a, 1974). Of the other 63 strains examined, 35 were cultured from the fossa navicularis of normal subjects and 28 from the vagina or urethra of female patients with normal urogenital tracts (Furness and others, 1973). Suspensions of 'NSU *Corynebacteria*' were prepared by

heavily inoculating Tween 80 agar plates (Furness and others, 1973) so that the growth was confluent after overnight incubation at 37°C. The bacteria were washed off the agar with 0.85 per cent. physiological saline and centrifuged at 2,000 G for 10 min. The supernatant was decanted and the cells washed twice and re-suspended in physiological saline. After breaking up any aggregates by sonication for 1 min. in a Raytheon Sonic Oscillator Model DF101 cooled with ice water, the concentration of cells was standardized on a Klett-Summerson Photoelectric Colorimeter at McFarland standard opacity tube number 3, which viable counts confirmed was equivalent to about 1×10^8 'NSU *Corynebacteria*' per ml. These saline suspensions were used to immunize rabbits, to prepare skin testing reagents, and as the antigens in complement-fixation tests.

IMMUNIZATION AND SKIN TESTING OF RABBITS

Four rabbits, each weighing 2.5 kg. were injected, two with the saline suspension and two with the saline suspension emulsified in Freund's incomplete adjuvant (Kallestad Labs., Inc., Minneapolis). The rabbits injected with saline suspensions of viable 'NSU *Corynebacteria*' received two subcutaneous injections of 0.25 ml., one on each side of the backbone. Those injected with adjuvant emulsions were given five subcutaneous injections of 0.2 ml., two on each side of the backbone and one in the nuchal region. The adjuvant emulsions were prepared by thoroughly mixing an equal volume of the saline suspension of viable 'NSU *Corynebacteria*' and Freund's incomplete adjuvant by repeated aspiration and ejection of the mixture through an 18 gauge needle attached to a 10-ml. hypodermic syringe.

Skin testing reagents were prepared by heating a known volume of the saline suspension at 100°C. for 5 min. to inactivate the 'NSU *Corynebacteria*'. Thereafter, the suspension was made up to volume with sterile distilled water. That the saline suspensions were sterile was confirmed by incubating for 5 days at 37°C. chocolate agar, blood agar, Tween 80 broth, nutrient agar, and thioglycollate broth inoculated with aliquots of the suspension. The rabbits were tested for hypersensitivity by the intradermal injection into their shaved flanks of the minimal volume of killed bacterial suspension which raised a tiny bleb.

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COMPLEMENT-FIXATION TESTS

The microtitre system (Microbiological Associates, Bethesda, Maryland) was used and the tests were performed in wells in auto-tray plastic plates (Canalco, Inc., Rockville, Maryland). Into each well was pipetted 0.025 ml. veronal buffered saline (Mayer, Osler, Bier, and Heidelberger, 1948); then 0.025 ml. serum inactivated at 56°C. for 30 min. was pipetted into the first well and 2-fold serial dilutions made using a microdiluter. Thereafter 0.025 ml. bacterial suspension containing 1×10^9 cells per ml. was added to each well followed by 0.025 ml. complement containing 3 MHD and the plates were covered with a plastic sheet. After incubation at 37°C. for 90 minutes, 0.05 ml. of a 0.5 per cent. sheep red blood cell suspension sensitized with 3 MHD haemolysin was added to each well and the plates re-incubated for 90 min. The endpoint was read as that dilution of serum which prevented haemolysis. In every test, controls were included to confirm that both the bacterial suspensions and the sera being examined for antibodies were not anti-complementary and that the complement had not lost its activity.

Results

IMMUNE RESPONSE IN RABBITS

Before immunization each rabbit was skin-tested and its serum examined for complement-fixing antibodies to the strain of 'NSU *Corynebacteria*' to be used for infection. Out of 26 rabbits, one had a titre of complement-fixing antibodies of 1/32 and another gave a slight delayed hypersensitivity reaction. To ascertain the significance of these observations the two rabbits were injected with

viable 'NSU *Corynebacteria*'. The antibody titre did not rise, and the rabbit with delayed hypersensitivity did not give a more marked response 7 weeks after injection, indicating that the reactions were non-specific. No reactions were detected in 24 animals. The total volume of the saline suspension or adjuvant emulsion used to inject a rabbit contained 5×10^7 viable organisms. No reaction was observed at the site of the injection of those rabbits injected with saline suspensions. In contrast, those injected with adjuvant developed at each site a hard indurated swelling up to 2 cm. diameter which suppurred. The exudate was sterile. In some animals the swellings started to appear within 4 to 6 days, while in others they were not apparent for at least 15 days.

RESPONSE TO SKIN-TESTING

7 weeks after injection the animals were bled and then skin-tested with a killed suspension of the homologous organism containing 1×10^8 cells. In each of the six groups of four rabbits, one animal gave a poor delayed hypersensitivity response compared to the other three. Of the rabbits giving minimal reactions, three had been injected with bacteria in adjuvant and three with saline suspensions. Therefore, this lack of hypersensitivity could not be attributed to the strain of 'NSU *Corynebacteria*' nor to the method of injection, but to variations in the ability of individual rabbits to become hypersensitive. Typical delayed hypersensitivity reactions are shown in the Figure.

FIGURE Delayed hypersensitivity reactions in sensitized rabbits 72 to 96 hrs after intradermal injection of (a) physiological saline control, (b) and (c) heat-killed suspensions of 'NSU *Corynebacteria*'

2 weeks after being skin-tested with the homologous strain, every rabbit was skin-tested with the other five epididymal strains. All cross-reacted. Then they were skin-tested with three commensals isolated from the fossa navicularis of normal individuals. Four of the six animals which reacted poorly to their homologous strain did not react to these commensals. Of the remaining eighteen rabbits, fourteen were hypersensitive to all three strains. Therefore, a further 32 strains isolated from the fossa navicularis and 28 from the vagina of normal subjects were tested for their ability to elicit delayed hypersensitivity reactions. Each of twenty rabbits was tested with three of the strains. Only one bacterial suspension did not elicit any skin reaction. However, although cross-reactions occurred, there were marked differences in the response of individual rabbits. In some, the skin reactions appeared in 48 hours. In others they took 4 to 5 days. Moreover, they varied in the time required for the inflammation to subside. It was noted that the first series of skin tests caused no apparent discomfort to the animals. However, the second and third series obviously caused irritation as the sites of the intradermal injections were badly scratched by many rabbits.

COMPLEMENT-FIXATION TESTS

Sera were obtained 7 weeks after injection and the titre of complement-fixing antibodies to the homologous strain ascertained. The sera from 21 rabbits were tested and none had detectable antibodies.

Discussion

Non-specific urethritis has many of the characteristics of an infection which is transmitted by sexual contact. The incubation period has been estimated to range from a few days to over a month with a peak at 2 to 3 weeks. The symptoms vary in severity and may last from one to several months. There is probably more than one cause of NSU. The condition has been attributed to *Chlamydia* (Wang and Grayston, 1970; Dunlop, Vaughan-Jackson, and Darougar, 1972; Oriel, Reeve, Powis, Miller, and Nicol, 1972), 'NSU *Corynebacteria*' (Furness and Csonka, 1966; Furness and others, 1971b) and T-mycoplasmas (Csonka, Williams, and Corse, 1966; Shepard, 1970). Reiter's disease may ensue in some cases.

The first episode of NSU usually responds to treatment with broad-spectrum antibiotics which indicates that an infectious agent is involved. However, relapses are fairly frequent and may not respond to antibiotic therapy, which is evidence that some of these subsequent episodes are not due solely to re-infection but rather to another cause which may be hypersensitivity. Grimble and Csonka (1955) pre-

pared a skin-testing reagent from the urethral discharge of patients with NSU. Delayed hypersensitivity reactions were elicited in 50 to 70 per cent. of patients with NSU after intradermal injection of this reagent, whereas only a small number of controls gave positive results. They concluded that there is a predominating cause for NSU, in all probability an infective agent.

Delayed hypersensitivity reactions could invariably be elicited after the subcutaneous injection of rabbits with viable 'NSU *Corynebacteria*', but the intensity of the skin reaction after the intradermal injection of skin testing suspensions of the homologous organism varied, which indicated that the rabbits differed in their ability to become hypersensitive. Those rabbits giving a good reaction to the homologous organisms generally cross-reacted with other strains of 'NSU *Corynebacteria*' isolated from the epididymis of patients with epididymitis and from the normal male and female urogenital tracts, whereas most of those rabbits giving a poor reaction did not cross-react with the latter. It took 2 to 5 days for the skin reaction to reach the maximum size and the period required for the inflammation to subside varied greatly in different animals.

No reaction was detected at the site of the injection in those animals injected with saline suspensions of viable 'NSU *Corynebacteria*'. In contrast, swelling and induration occurred in those injected with the adjuvant emulsions. This indicates that the NSU *Corynebacteria* retained *in situ* elicited a delayed hypersensitivity reaction when the animals became hypersensitive. As such, this response is an indicator of the time required to induce hypersensitivity. The swelling began to appear within 4 to 6 days in some animals, indicating that it is possible for hypersensitivity to be stimulated very rapidly.

These results may explain the manifestations in those cases of NSU and Reiter's disease in which the disease can be attributed to 'NSU *Corynebacteria*'. The isolation of 'NSU *Corynebacteria*' from the epididymis of patients with acute epididymitis is evidence that some species are capable of initiating an infection (Furness and others, 1971a, 1974). However, NSU often follows gonorrhoea and it is possible that, in these circumstances, 'NSU *Corynebacteria*' which are commensals, can be responsible for a secondary infection of the urethra. Thereafter, depending on the severity of the infection, the inherent tendency of the patient to become hypersensitive, and the intensity of the hypersensitivity, either this initial infection or repeated infections may induce delayed hypersensitivity which ultimately leads to clinical manifestations. Antibiotic therapy would be effective until the patient became hyper-