Reduced resistance to Pseudomonas septicaemia in diabetic mice

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SUMMARY

Antibacterial resistance in a diabetic state was studied using experimental Pseudomonas infection in streptozotocin (SZ) induced diabetic mice. The results obtained were as follows: (1) there was no difference in acute death rate between normal and diabetic mice when infected with *Pseudomonas aeruginosa*. However, a significant increase in the number of bacteria in the kidney and liver occurred at a later stage of infection in diabetic mice. (2) Active immunization with a phenolized vaccine resulted in 100% survival in either normal or diabetic mice; otherwise challenge was lethal. However, the organs examined in diabetic vaccinated mice contained distinctly increased numbers of bacteria as compared with normal vaccinated mice 7 days after infection. (3) There were no significant differences in antibody titre between normal and diabetic mice after infection, but passive protection with immune serum from diabetic vaccinated mice was less effective than that from vaccinated mice. Furthermore, immune serum from normal vaccinated mice exerted protective action less efficiently in diabetic recipients than in normal recipients. (4) The bactericidal effect of peripheral whole blood was apparently lower in diabetic mice than in normal mice. (5) Treatment with insulin restored such reduced resistance to Pseudomonas infection in diabetic mice. These findings suggest that the decreased resistance to Pseudomonas infection in diabetic mice should be ascribed to impaired function of antibody, abnormalities in phagocytic cells and disturbed microcirculation caused by the insulin-deficient state.

INTRODUCTION

Diabetic patients are known to be more susceptible to bacterial infection. Meanwhile, the incidence of *Pseudomonas aeruginosa* infection has increased in recent years (Finland, 1973; Bennett, 1974). *Pseudomonas aeruginosa* generally invades the immunosuppressed hosts because of low virulence in healthy hosts. Our previous experiments have shown that in SZ-induced diabetic mice, the immune responses (antibody formation and delayed-type hypersensitivity) to SRBC were depressed (Ishibashi *et al.*, 1980). In particular, antibody formation was more severely depressed than delayed-type hypersensitivity response. Since it is generally accepted that resistance to Pseudomonas infection depends on antibody-mediated immunity (Jones, 1968; Homma, 1971; Bjornson & Michael, 1970, 1971; Young & Armstrong, 1972; Reynolds, Kazmierowski & Newball, 1975), it is of interest to know the course of experimental Pseudomonas infection in SZ-induced diabetic mice. Thus the present studies were designed to compare the course of Pseudomonas infection and the development of acquired resistance in normal and SZ-induced diabetic mice.

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MATERIALS AND METHODS

Mice. Female closed-colony-bred CF_1 mice were obtained from the Animal Supply Centre, Kyushu University. Mice were maintained under conventional conditions and were 8 weeks old at the beginning of the experiments. In each experiment, mice were divided into two groups. One group of mice was injected with SZ, the other group served as a contemporaneous control.

Induction of streptozotocin diabetes. Streptozotocin (lot no. 60273–2) was obtained from the Upjohn Co. (Kalamazoo, Michigan, USA). Induction of diabetes with SZ was carried out as described previously (Ishibashi *et al.*, 1980). Mice were given an intravenous injection of SZ (140 mg/kg of body weight). Only mice with glycosuria of 0·5% or higher 4 to 6 weeks after SZ had been injected were used as diabetic mice in the experiment. However, all diabetic mice showed no ketonuria at the time of infection. Blood glucose determination was performed by the glucose–oxidase method as described previously (Ishibashi *et al.*, 1980).

Bacteria. Pseudomonas aeruginosa (strain NC-5) was kindly provided by Dr J. Y. Homma (Institute of Medical Science, University of Tokyo, Japan). This strain was highly virulent for mice and produced neither exotoxin, protease, nor lecithinase (Kobayashi, 1971). The intravenous mean lethal dose (LD_{50}) for CF_1 mice was found to be $5·8 \times 10^6$ viable bacteria without use of mucin during the last 2 years.

Infection of mice. Bacterial suspensions were made as described previously and viable bacteria were enumerated by plate count on trypticase soy agar (Ishibashi *et al.*, 1978). Mice were infected i.v. with 0·2 ml of bacterial suspension adjusted to desired concentration. Challenged mice were observed routinely for 7 days following infection.

Enumeration of in vivo *bacterial population.* The course of infection was followed quantitatively by bacteria counts in various organs. The liver, spleen and kidneys were cultured as described previously (Ishibashi *et al.*, 1978). The viable counts in each organ were expressed in $\log_{10}$ units.

Bactericidal assay of peripheral blood. Heparinized blood (0·5 ml) obtained by heart puncture was placed in a polypropylene tube (2054, Falcon Plastics, Los Angeles, California) and 0·2 ml of the Pseudomonas suspension added ($2–3 \times 10^5$ *P. aeruginosa*/0·1 ml). The tube containing this mixture were rotated (150 r.p.m.) on a Gyrotory (Shakermodel G2, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey) at 37°C for 3 hr. One and 3 hr after incubation, 0·1 ml of the samples was removed, serially diluted in distilled water and plated on trypticase soy agar. Bacterial count was carried out after incubation at 37°C for 24 hr.

Vaccination. A phenolized whole cell vaccine was prepared according to the method described by Pierson, Johnson & Feller (1976). The concentration of vaccine was 6×10^8 dead bacteria per ml. The vaccine was administered intraperitoneally. Immune serum was prepared in normal or diabetic mice by giving 0·2 ml of vaccine i.p. and obtained 12 days after vaccination. They were stored at $-20°C$ until use.

Antibody determination. Antibody response was measured by passive haemagglutination using sheep erythrocytes coated with the protein moiety (OEP) of the endotoxin of *P. aeruginosa* (Tomiyama *et al.*, 1973; Homma, 1971). A kit of this passive haemagglutination was kindly provided by Dr C. Abe (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Haemagglutination titres were determined by the microtitre method. The titre was recorded as the reciprocal of the highest dilution of serum showing positive haemagglutination.

Insulin. Insulin (1·5 iu; Novo Lente, Denmark) was administered subcutaneously every day from 5 days before infection up to the day of killing.

Statistical determination. The death rate between experimental groups was analysed according to the method of Peto (1974). The data on the growth curve of bacteria were analysed by Student's *t*-test. The data on the number of bacteria on day 7 following survival studies were analysed according to the method of Mann & Whitney (1947) because we could not detect any viable bacteria in considerable numbers of infected mice.

RESULTS

Primary resistance to Pseudomonas septicaemia in diabetic mice

Firstly, mortality studies were performed in diabetic mice and contemporaneous normal controls

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after infection of varying doses of *P. aeruginosa*. Table 1 shows that no differences between the mortality of diabetic and normal mice were observed at any infecting doses. In the next experiment, the course of infection was followed by quantitative enumeration of bacteria in the liver, spleen and kidneys. As shown in Fig. 1, the numbers of bacteria in tissues were steadily decreased after infection in normal controls. Whereas the number of bacteria in diabetic mice was decreased during the 6 hr of infection similarly to that seen in normal controls, it was increased thereafter. In particular, the increase in the number of bacteria was remarkable in the liver and kidneys.

Table 1. Per cent survival of normal and diabetic mice infected with varying doses of *P. aeruginosa*

Group	Challenge dose	Per cent survival 7 days after infection
Diabetic	1×10^7	10
	5×10^6	60
	5×10^5	100
Control	1×10^7	10
	5×10^6	70
	5×10^5	100

CF$_1$ mice were injected i.v. with SZ (140 mg/dl) 6 weeks before testing. Normal and diabetic mice were concurrently infected i.v. with the doses indicated of *P. aeruginosa*. Each group consisted of 10 mice.
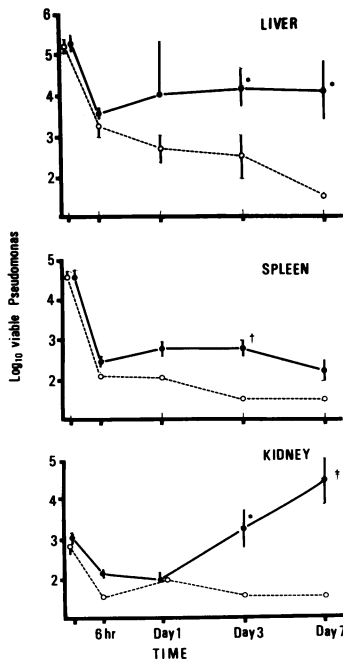

Fig. 1. The fate of *P. aeruginosa* in normal and diabetic mice. Normal (o – – – o) and diabetic (●———●) mice 6 weeks after SZ injection were concurrently infected i.v. with a sublethal dose of 2.4×10^6 *P. aeruginosa*. Each point represents the mean of five mice ± s.d. Blood glucose concentrations were 147 ± 5.9 mg/dl in normal and 442 ± 17.2 mg/dl in diabetic mice when determined in the five mice randomly selected from each group a day before infection. * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.005$ in comparison with control.

Furthermore, gross examination revealed many localized lesions in the kidneys, occasionally in the liver in diabetic mice.

Effect of active immunization with Pseudomonas vaccine in diabetic mice
Both groups of normal controls and diabetic mice were divided into three subgroups. The first groups of normal and diabetic mice (groups 1 and 4) served as controls without vaccination. The second groups (groups 2 and 5) were given 0·02 ml of vaccine and the third groups (groups 3 and 6) received 0·1 ml of vaccine. All mice were infected concurrently i.v. with $1·7 \times 10^7$ *P. aeruginosa*. Survival was 100% in both groups of normal and diabetic mice which had received either 0·1 or 0·02 ml of vaccine whereas all mice died in both normal and diabetic mice without vaccination (groups 1 and 4). The bacterial counts in survivors clearly showed that all the organs examined in diabetic vaccinated mice contained distinctly increased numbers of bacteria as compared with those of normal vaccinated mice (Table 2.) However, the effect of 0·1 ml of vaccine did not differ from that of 0·02 ml in either group of normal and diabetic mice.

In the next experiments, the course of infection with a low dose of challenge inoculum was examined after immunization with 0·2 ml of vaccine. Normal and normal vaccinated mice were infected i.v. with 7×10^6 *P. aeruginosa*. Survival was 40% in normal controls and 100% in normal vaccinated mice. Secondly, diabetic controls and diabetic vaccinated mice were infected i.v. with 8×10^6 *P. aeruginosa*. Survival was 40% in diabetic mice and 100% in diabetic vaccinated mice. The results of bacterial counts are presented in Table 3. The numbers of bacteria in normal controls were higher than those in normal vaccinated mice although the difference was not statistically significant. On the other hand, the liver and kidneys in diabetic vaccinated mice contained comparable numbers of bacteria to diabetic controls.

Antibody response to Pseudomonas infection in diabetic mice
Normal mice and diabetic mice with or without insulin were infected with 3×10^6 *P. aeruginosa*. Mice were bled by cutting the tail vein on days 3, 7 and 10 after infection. The results are shown in Fig. 2. The antibody titres in diabetic mice with or without insulin treatment were slightly higher than in normal controls. However, no significant differences in the antibody titres were observed among three experimental groups.

Table 2. The fate of *P. aeruginosa* in the liver, spleen and kidneys of normal and diabetic mice given vaccine

Group	Dose of vaccine (ml)	No. of mice	Mean of $\log_{10}$ viable Pseudomonas		
			Liver	Spleen	Kidneys
Normal					
Group 2	0·02	10	1·14	0·17	1·93
Group 3	0·1	10	1·57	0	1·1
Diabetic					
Group 5	0·02	10	2·97	1·31	3·76
Group 6	0·1	10	3·45	1·18	4·93

Normal and diabetic mice which had received SZ 6 weeks before were injected i.p. with the doses indicated of Pseudomonas vaccine. Twelve days later mice were injected i.v. with $1·7 \times 10^7$ *P. aeruginosa*. Blood glucose concentrations were $149 \pm 1·6$ mg/dl in controls and $482 \pm 41·4$ mg/dl in diabetics when determined in the five mice randomly selected from each group before vaccination. Bacterial counts were performed in the liver, spleen and kidneys 7 days after infection. When viable bacteria were not detected in the organ, it was represented as 0 and the arithmetric mean was calculated. The difference between group 3 and 6 was significant to $P < 0·002$ in the liver and kidneys.

Table 3. The fate of *P. aeruginosa* in the liver, spleen and kidneys of normal and diabetic mice given vaccine

| Group | No. of mice | Mean of $\log_{10}$ viable Pseudomonas | | |
		Liver	Spleen	Kidneys
Normal	5	2·44	0·74	2·48
Normal vaccinated	5	0·8	0·68	1·04
Diabetic	5	2·72	1·92	5·12
Diabetic vaccinated	5	2·08	0·68	3·64

Normal controls and normal vaccinated mice which had been given 0·2 ml of vaccine 12 days before were infected i.v. with 7×10^6 *P. aeruginosa*. In a separate experiment, diabetic controls and diabetic mice given 0·2 ml of vaccine 12 days before were infected i.v. with 8×10^6 *P. aeruginosa*. Bacterial counts 7 days after infection are shown. Diabetic mice 6 weeks after SZ injection were used. Blood glucose concentration was $412 \pm 30·6$ mg/dl when determined 1 day before vaccination. There was no significant difference in the number of bacteria between vaccinated and non-vaccinated groups in either normal or diabetic mice.
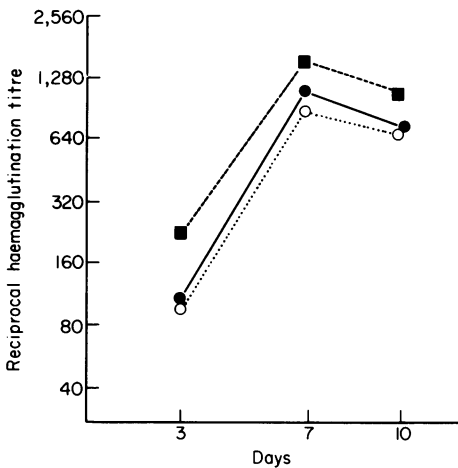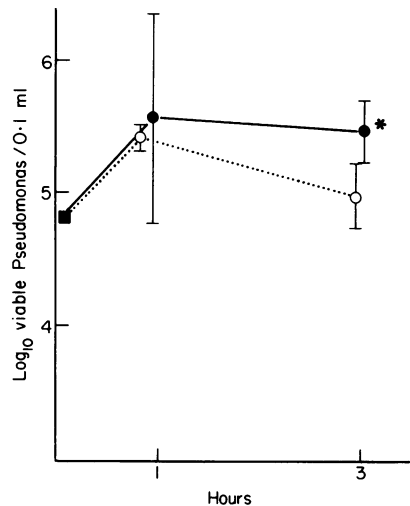
Fig. 2.   Fig. 3.

Fig. 2. Antibody response to Pseudomonas infection in diabetic mice. Normal controls (o – – – o) and diabetic mice with (■ – – – ■) or without (●——●) insulin treatment were infected i.v. with 3×10^6 *P. aeruginosa*. Each point represents the geometric mean titre of five mice. Blood glucose levels were $148 \pm 3·6$ mg/dl in normal controls, $588 \pm 40·9$ mg/dl in diabetics and $238 \pm 44·9$ mg/dl in diabetics treated with insulin when determined a day before infection.

Fig. 3. Bactericidal activity of peripheral blood of diabetic mice against *P. aeruginosa*. One-half of a millilitre of blood and 0·2 ml of the Pseudomonas suspension ($4·5 \times 10^5$ bacteria) were mixed and incubated for 3 hr. Blood glucose concentrations were $159 \pm 9·2$ mg/dl in normal (o – – – o) and $594 \pm 31·2$ mg/dl in diabetic (●——●) mice. The WBC counts were 7, 850 ± 640 (MN 85·2% and PMN 14·8%) in normal mice, $9,160 \pm 1,490$ (MN 71·5% and PMN 28·5%) in diabetics. Each point represents the mean of five $\pm$ s.d. * $P < 0·02$ in comparison with control.

Protective activity of immune serum obtained from diabetic mice given vaccine

Experiments were performed to compare the protective activity of immune serum obtained from diabetic vaccinated mice with that from normal vaccinated mice. Normal recipients were infected with lethal doses of *P. aeruginosa* immediately after serum transfer. The results in Table 4 show that survival was 90% in normal mice given normal immune serum, whereas it was 40% in mice which had received diabetic immune serum. All normal controls died. In the subsequent experiments, the efficacy of passive protection by immune serum from normal vaccinated mice was examined in normal and diabetic recipients. Normal immune serum was the same as used in the foregoing experiment. The results showed that survival was 100% in normal mice given immune serum. In contrast, it was 50% in diabetic mice in spite of the passive transfer of the same immune serum. The bacterial counts in the survivors in Table 5 showed that the numbers of bacteria in all organs examined in diabetic mice were much higher than those of normal animals.

Table 4. Per cent survival of normal mice which had received immune serum obtained from normal and diabetic mice given vaccine

Group	Immune serum transferred	Per cent survival 7 days after infection
1	Immune serum from normal vaccinated mice	90
2	Immune serum from diabetic vaccinated mice	40
3	Control without serum	0

Normal and diabetic mice which had received SZ 4 weeks before were injected i.p. with 0·2 ml of Pseudomonas vaccine; 12 days later they were killed for serum collection. Blood glucose levels were $161 \pm 6·1$ mg/dl in normal immune serum and $661 \pm 55·1$ mg/dl in diabetic immune serum. Both immune sera from either normal or diabetic vaccinated mice were found to give a titre of 1:2,560. Normal recipient mice were injected i.v. with 0·2 ml of each immune serum and subsequently infected i.v. with $1·4 \times 10^7$ *P. aeruginosa*. The difference in the death rate between groups 1 and 2 was significant to $P < 0·05$.

Table 5. The fate of *P. aeruginosa* in the liver, spleen and kidneys of normal and diabetic mice which had received immune serum

Group	No. of mice	Mean of $\log_{10}$ viable Pseudomonas		
		Liver	Spleen	Kidneys
Normal	10	0·99	0·68	0·76
Diabetic	6	3·67	1·43	5·48

Normal and diabetic mice which had received SZ 5 weeks previously were injected i.v. with 0·2 ml of immune serum from normal vaccinated mice and subsequently infected i.v. with $1·3 \times 10^7$ *P. aeruginosa*. Blood glucose concentrations were $164 \pm 6·8$ mg/dl in normal recipients, 552 ± 34 mg/dl in diabetic recipients a day before infection. The difference in the numbers of bacteria between normal and diabetic recipients was significant to $P < 0·002$ in the liver and kidneys.

Bactericidal activity of peripheral blood of diabetic mice

The *in vitro* bactericidal activity of peripheral blood obtained from diabetic mice 4 weeks after SZ injection was compared with that of normal controls. As can be seen in Fig. 3, the number of viable bacteria after incubation for 3 hr in the blood from normal mice was apparently lower than that of diabetic mice.

Influence of insulin treatment on resistance to Pseudomonas infection in diabetic mice

Diabetic mice with or without insulin treatment and normal controls were concurrently infected i.v. with 8×10^6 *P. aeruginosa*. Survival was 80% in both normal mice and diabetic mice and 73% in diabetics treated with insulin. The results in Table 6 show that the number of bacteria in diabetic mice treated with insulin was reduced to the level of normal controls.

Table 6. The fate of *P. aeruginosa* in the liver, spleen and kidneys of diabetic mice treated with insulin

| Group | No. of mice | Mean of $\log_{10}$ viable Pseudomonas | | |
		Liver	Spleen	Kidneys
Normal	8	0·68	0·73	1·68
Diabetic	8	3·61	1·85	5·32
Diabetic + insulin	8	1·05	1·44	0·81

Diabetic mice which had received SZ 6 weeks before infection were divided into two groups. One group of mice served as diabetic controls, the other group was injected with 1·5 i.u. of insulin every day from 5 days before infection up to the day of killing. Mice were infected i.v. with 8×10^6 *P. aeruginosa*. Blood glucose concentrations were $172 \pm 2 \cdot 8$ mg/dl in normal controls, 613 ± 37 mg/dl in diabetic controls and $64 \pm 4 \cdot 0$ mg/dl in diabetics treated with insulin when determined without fasting 5 hr after insulin injection 1 day before infection. Survivors were killed 7 days after infection. The difference in the number of bacteria between diabetics and diabetics treated with insulin was significant to $P < 0 \cdot 002$ in the liver and kidneys.

DISCUSSION

Bartell, Orr & Garcia (1968) stated that the death of 80 to 100% of mice infected with a large dose of *P. aeruginosa* occurred in 24 to 48 hr. Gorrill (1965) reported that 25 to 30% of mice infected with doses in the order of 10^8 bacilli died. After deaths in the first 2 days, which were attributed to the action of endotoxin, there were very few deaths in the next 10 weeks despite severe kidney lesions. Our previous studies also showed that intravenous inoculation with *P. aeruginosa* (NC-5) in mice caused early toxic death within 2 to 3 days, followed by sporadic late death induced by localized lesions in the kidneys (Ishibashi *et al.*, 1978; Harada *et al.*, 1979). Such early deaths occurred prior to an increase in the number of bacteria. The present studies showed that there was no difference in death rate between normal and diabetic mice. We also showed that increased multiplication of *in vivo* bacteria and increased formation of localized lesions occurred at later stages – day 7 in diabetic mice. It has been reported by several investigators that mice were protected from Pseudomonas infection by active immunization with vaccine (Fitzpatrick & Girard, 1968; Pierson *et al.*, 1976). Moreover, it has been shown by many investigators that immune serum from vaccinated mice gives passive protection to normal mice against lethal Pseudomonas infection (Millican & Rust, 1960; Jones, Lilly & Lowbury, 1971; Pierson *et al.*, 1976; Ishibashi *et al.*, 1978). In the present experiments, a single administration of vaccine resulted in 100% survival in either normal or diabetic mice.

However, the bacterial count showed that the effect of vaccination was less efficient in diabetic mice than in normal mice. Since antibody production to SRBC was markedly depressed in diabetic mice (Ishibashi *et al.*, 1980), we supposed that the increase in the number of bacteria at later stages in diabetic mice would be due to depressed antibody response to *P. aeruginosa*. However, there were no significant differences in antibody titre between normal controls and diabetic mice. In contrast, the protective activity of immune serum from diabetic vaccinated mice was clearly lower than that from normal vaccinated mice. Further studies are needed to investigate the class and avidity of antibody produced to *P. aeruginosa* in diabetic mice. Since immune serum protects bacteria and inhibits their growth in tissues, it seems that the resistance to *P. aeruginosa* was not based on cell-mediated immunity, but on antibody-mediated immunity.

Numerous investigators have shown that the increased susceptibility of diabetics to infection should be ascribed to abnormalities in polymorphonuclear leucocyte (PMN) function in man and animals (Sheldon & Bauer, 1959; Wertman & Henney, 1962; Perillie, Nolan & Finch, 1962; Bybee & Rogers, 1964; Mowat & Baum, 1971; Bagdade, Nielson & Bulger, 1972; Nolan, Beaty & Bagdade, 1978). Bjornson & Michael (1972) and Young & Armstrong (1972) have shown that polymorphonuclear leucocytes are essential in the resistance of animals to experimental Pseudomonas infection. Our results showed that protection exerted by normal immune serum was less efficient in diabetic mice than in normal controls. Furthermore, the *in vitro* bactericidal assay showed that peripheral blood from diabetic mice achieved a less efficient bactericidal effect than that from normal controls. These findings indicate that malfunction of leucocytes in diabetic mice should be partly responsible for reduced resistance to infection.

In the present experiments, treatment with insulin completely restored the decreased resistance to Pseudomonas infection in diabetic mice. Recent investigations demonstrated that circulating leucocytes including lymphocytes, monocytes and granulocytes possess specific receptors for insulin (Gavin, Buell & Roth, 1972; Gavin *et al.*, 1973; Schwartz *et al.*, 1975; Olefsky & Reaven, 1976; Fussganger *et al.*, 1976). Furthermore, it has been reported by several investigators that insulin has various influences on immunological function (Mowat & Baum, 1971; Strom, Bear & Carpenter, 1975; Rhodes, 1975; Bar, Kahn & Koren, 1977). Numerous investigators also reported that diabetic angiopathy such as nephropathy developed in experimental diabetic animals (Lundback *et al.*, 1967; Osterby, 1975). In fact, we observed that the kidneys of diabetic mice were enlarged and, after insulin treatment, their size returned to normal. It therefore seemed that insulin caused a reversal of functional abnormalities in diabetic leucocytes and improvement of microcirculation in tissues. Thus it was evident that the impaired host defence to *P. aeruginosa* in SZ-induced diabetics appeared complex and that both immunological and non-immunological factors play a role in the reduced resistance to infection.

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