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Evidence for suppressor cell activity associated with depression of contact sensitivity in *Pseudomonas aeruginosa* infected mice

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SUMMARY

Pseudomonas aeruginosa infection depresses contact sensitivity to 2-phenyl-4-ethoximethylene-oxazolone (oxazolone), and enhances the antibody response to sheep erythrocytes (SRBC) in the mouse.

Anti-oxazolone antibody titres were found not to be significantly different in infected and uninfected animals; thus, the major circulating classes of antibodies do not seem to be responsible for the observed depression of skin reactivity.

Low dose (20 mg/Kg) cyclophosphamide (CY) pretreatment induced a further potentiation of antibody response to SRBC, and prevented depression of contact sensitivity in infected mice.

On the other hand, when infected animals were pretreated with high doses (200 mg/Kg) of CY, antibody production was completely suppressed, whereas contact sensitivity was unaffected.

Since CY treatment is known to selectively inhibit B lymphocytes, and since it can abrogate the infection-induced depression of reactivity to oxazolone, it is suggested that suppressor cells, which may have B-cell characteristics, are stimulated during *P. aeruginosa* infection in the mouse.

INTRODUCTION

Previously reported experiments from our laboratory have shown that *Pseudomonas aeruginosa* infection in mice induces both a marked depression of contact sensitivity to 2-phenyl-4-ethoximethylene-oxazolone (oxazolone) and a significant enhancement of humoral response to sheep erythrocytes (SRBC), horse erythrocytes, and *E. coli* lipopolysaccharide (Campa, Garzelli & Falcone, 1975).

This reciprocal relationship between the humoral and the cell-mediated immunity has been described with other bacteria. For example, it has been demonstrated that the systemic injection of killed *Corynebacterium parvum* can increase the phagocytic capability of spleen macrophages, which then amplify the B-cell response on the one hand, and deplete delayed-type-hypersensitivity (DTH)-reactive T cells on the other (Howard, Scott & Christie, 1973; Scott, 1974).

However, we found that injection of killed *P. aeruginosa* in mice potentiated antibody production, whilst it did not impair contact sensitivity (Campa, Garzelli & Falcone, 1975). Thus, the interference of *P. aeruginosa* on the immune response seems to involve a different mechanism.

Since antibodies are known to depress the cell-mediated response against the same antigen (Uhr & Möller, 1968), anti-oxazolone antibodies could also be increased as a consequence of the observed, infection-induced potentiation of humoral response, and could thus mediate the depression of skin reactivity to oxazolone. However, we report here that no significant differences in such antibody titres were found between infected and uninfected animals.

To investigate the possibility that suppressor cells could be specifically stimulated by *P. aeruginosa* infection, cyclophosphamide (CY) was administered to infected mice before either sensitization with oxazolone or immunization with SRBC. The rationale of this approach is based on the observation that

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CY affects preferentially B rather than T lymphocytes and inhibits the activity of the cells which suppress delayed hypersensitivity reactions (Turk, Parker & Poulter, 1972; Katz *et al.*, 1974; Polak & Turk, 1974; Zembala & Asherson, 1976).

Evidence is here presented that *P. aeruginosa* infection does interact with suppressor lymphocytes in the mouse.

MATERIALS AND METHODS

Animals. $C_{57}BL/6$ mice of both sexes, aged 8–12 weeks, were used throughout. In each experiment the animals were randomly allocated to the different groups.

Bacteria. A strain of *P. aeruginosa* isolated from clinical specimens was used. A stock culture, obtained after several passages on synthetic medium (Stayner, Palleroni & Doudoroff, 1966), was stored in small aliquots at −20°C until use.

Infection of mice. Inocula were prepared as previously described (Campa *et al.*, 1975) to give final suspensions containing approximately 120×10^6 organisms per ml which corresponds to the median lethal dose (LD_{50}). Mice were inoculated intraperitoneally with 0·2 LD_{50} in 0·5 ml, since in previous experiments this was the lowest infective dose which gave detectable depression of contact sensitivity (Campa *et al.*, 1975).

Sensitization, challenge and quantification. Mice were anaesthetized by intraperitoneal administration of tribromoethanol in amylene hydrate (Avertin, Wintrop, New York) and usually sensitized 24 hr after infection, by applying 0·2 ml of 1% (unless otherwise stated) solution of 2-phenyl-4-ethoximethylene-oxazolone (oxazolone; British Drug Houses, Poole) in absolute ethanol to the skin of the abdomen. The challenge was performed at 6 or 4 days after sensitization (unless otherwise indicated) by painting both sides of the ears with a drop of 1% oxazolone in olive oil, and the quantification was made by measuring, with a micrometer, the increase in ear thickness 24 hr later ($1\ u = 1\ cm\times10^{-3}$).

Immune response to SRBC. Sheep erythrocytes (SRBC), obtained from the same animal throughout these studies, were suspended in Alsever's solution. Mice were i.v. inoculated 24 hr after infection with 80×10^6 SRBC which had been washed four times in 10 vol. of sterile saline. Four days after SRBC administration, all mice were killed, and their sera were individually assayed for anti-SRBC haemoagglutinins and haemolysins, while the spleens were tested for direct plaque-forming cells (PFC), as previously described (Bendinelli, Toniolo & Campa, 1975).

Anti-oxazolone antibodies. Mice sensitized with 1% oxazolone were sacrificed 6 days after sensitization; each serum was adsorbed with SRBC and assayed for anti-oxazolone antibodies using oxazolone-coated SRBC, obtained by mixing oxazolone at a final concentration of 0·163 mg/ml with an initial suspension of 5% SRBC, as described by Askenase & Asherson (1972).

Cyclophosphamide treatment. Cyclophosphamide (CY; Endoxan-Asta, Astawerke AG, Brackwede, Germany), dissolved in sterile phosphate-buffered saline immediately before use, was injected intraperitoneally as a single dose of 200 mg/Kg 2 days before immunization or, for the dose of 20 mg/Kg, 1 day before immunization.

Statistical analysis. Data are expressed as the geometric mean ± 95% confidence limits unless otherwise indicated. Student's *t*-test was used to compare the differences between the means.

RESULTS

We have recently reported that mice infected with *P. aeruginosa* 24 hr before sensitization with 1% oxazolone show a marked depression of contact sensitivity (Campa *et al.*, 1975). Pilot experiments were performed to investigate the relationship between the dose of oxazolone and the degree of depression.

When groups of normal mice and mice infected 24 hr previously, were sensitized with varying doses of oxazolone (0·1, 0·5, 1, 2, and 3%) and challenged 6 days later, contact sensitivity plateaued at 2% oxazolone both in normal and in infected mice. The response in infected mice was lower than in controls at all tested doses, even though the difference reached statistical significance ($P<0·01$) only in the case of the mice sensitized with 1% oxazolone (Fig. 1). This dose was therefore used in all subsequent experiments.

The effect of varying the interval between infection and sensitization was also studied. In mice sensitized with 1% oxazolone and challenged 6 days later, contact sensitivity was significantly depressed ($P<0·02$) in those mice infected on the same day of sensitization. When infection preceded sensitization, significant depression ($P<0·01$) of contact sensitivity was observed only if the period between the two events was 1 day or 2 days and not 3 days or 4 days. When, on the other hand, infection followed sensitization, in no cases was contact sensitivity depressed (Fig. 2). Since maximal depression was evident in the mice infected 24 hr before sensitization, this schedule was maintained throughout our experiments.

The results of a typical experiment are reported in Table 1, mice infected 24 hr before sensitization

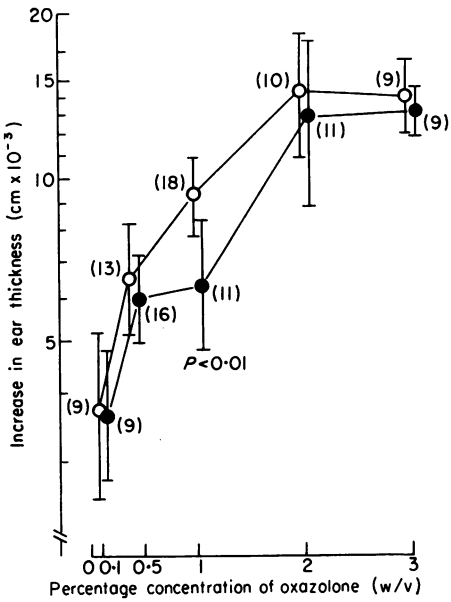

FIG. 1. Increase in ear thickness measured 6 days after sensitization with varying doses of oxazolone in infected (●——●) and uninfected (○——○) mice. Vertical bars indicate the 95% confidence limits; the number of animals is given in parentheses.

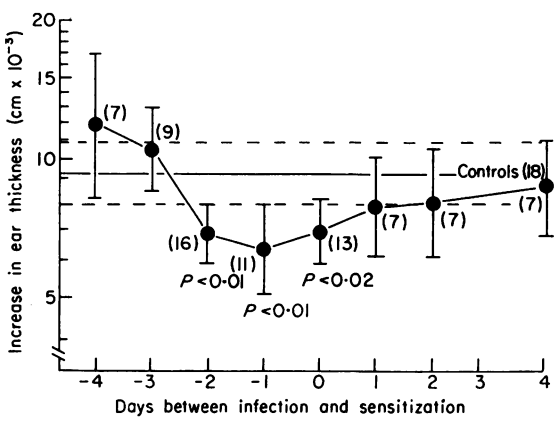

FIG. 2. Effect of varying the time-interval between infection and sensitization on the increase in ear thickness. In all groups of mice sensitization was performed on day 0, and challenge was done 6 days later; the other numerals on the abscissae refer to the day of infection. Horizontal lines are the geometric mean and 95% confidence limits of uninfected controls. Vertical bars indicate the 95% confidence limits, and *P* values refer to comparison (*t*-test) with the reported controls. The number of animals is given in parentheses.

with 1% oxazolone and challenged 6 days later were efficiently and significantly depressed ($P<0{\cdot}005$) when compared with uninfected sensitized animals.

To test the possibility that the depression of contact sensitivity might be due to high levels of anti-oxazolone antibodies, we assayed the serum antibody titres in mice infected with 0·2 LD_{50}, 6 days after sensitization. The anti-oxazolone antibody titre (expressed as in Table 4) was $6{\cdot}0\pm0{\cdot}7$ in twenty infected animals, whereas it was $5{\cdot}7\pm0{\cdot}8$ in twenty uninfected controls; the difference between the means was not statistically significant.

On the other hand, it has been recently shown (de Hurtado, Ovary & Osler, 1975) that very sensitive procedures are needed to detect low concentrations of antibodies against chemical contactants. Thus,

TABLE 1. Effect of *P. aeruginosa* infection on contact sensitivity in mice challenged 4 or 6 days after sensitization

Infection	Increase in ear thickness			
	No. of mice	At 4 days	No. of mice	At 6 days
	14	7·85 (6·70–9·20)	12	9·44 (7·92–11·25)
0·2 LD_{50}	12	6·05* (4·87–7·51)	11	6·25† (4·95–7·89)

Data expressed as geometric mean; the 95% confidence limits are given in parentheses.
* $P<0·05$
† $P<0·005$
} when compared with respective uninfected controls.

differences in anti-oxazolone antibody levels between infected and uninfected animals might be apparent at higher antibody titres than those detectable by the direct haemoagglutination technique which we adopted.

The response to oxazolone was then assessed in infected animals 4 days after sensitization. At this time, skin reactivity is well developed (Fig. 3), whereas antibody production against oxazolone can be assumed still to be very poor, on the grounds that, following sensitization, T cells are activated to peak proliferation by day 4, whilst B cells peak later, at day 8 (Davies *et al.*, 1969; de Sousa & Parrot, 1969). We found that at 4 days ear swelling was significantly depressed ($P<0·05$) in infected mice in comparison with their uninfected littermates (Table 1).

Since CY pretreatment is known to enhance contact sensitivity (Turk *et al.*, 1972; Turk & Parker, 1973), an attempt was made to abrogate or to prevent the development of the depression of contact sensitivity by this treatment. When mice were pretreated with either 20 mg/Kg or 200 mg/Kg CY i.p. and challenged 4 days (Table 2) or 6 days (Table 3) after sensitization, contact sensitivity was found no longer to be depressed in infected mice but slightly enhanced, even though this difference did not attain statistical significance. We note that in all pretreated animals the ear swelling was the same or slightly greater as in the uninfected, non-pretreated controls. That is to say, the ear swelling of all pretreated

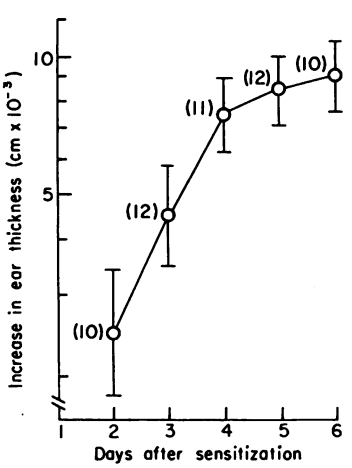

FIG. 3. Effect of different time-interval between sensitization and challenge on the increase in ear thickness for normal mice. Vertical bars indicate the 95% confidence limits of the geometric mean; the number of animals is given in parentheses.

TABLE 2. Effect of cyclophosphamide pretreatment on contact sensitivity of uninfected and infected mice 4 days after sensitization

No. of mice	Cyclophosphamide pretreatment	Infection	Increase in ear thickness
9	20 mg/Kg	—	7·99 (6·04–10·60)
9	20 mg/Kg	0·2 LD_{50}	9·61 (8·22–11·25)
12	200 mg/Kg	—	7·90 (6·51–9·59)
10	200 mg/Kg	0·2 LD_{50}	8·37 (5·58–12·56)

Data expressed as in Table 1.

TABLE 3. Effect of cyclophosphamide pretreatment on contact sensitivity of uninfected and infected mice 6 days after sensitization

No. of mice	Cyclophosphamide pretreatment	Infection	Increase in ear thickness
10	20 mg/Kg	—	9·64 (7·50–12·39)
10	20 mg/Kg	0·2 LD_{50}	10·79 (9·24–12·59)
13	200 mg/Kg	—	10·82 (8·73–13·40)
10	200 mg/Kg	0·2 LD_{50}	12·48 (10·48–14·86)

Data expressed as in Table 1.

mice was significantly greater than in infected, non-pretreated animals (comparing Tables 2 and 3 with Table 1).

The effect of CY pretreatment on antibody response to SRBC is shown in Table 4. Infection alone induced a significant enhancement ($P<0·02$, at least) both in PFC (expressed per 10^6 spleen cells) and in haemoagglutinin and haemolysin titres. Pretreatment of mice with 20 mg/Kg CY led to an increase, though non-significant, in PFC and antibody titres over controls. Infection of mice so pretreated, however, gave rise to higher values, higher even than the infected, unpretreated animals. Furthermore, high doses of CY depress antibody response (by all three parameters) both in uninfected and in infected animals, though in the latter group a low residual response was observed.

DISCUSSION

We have previously reported that *P. aeruginosa* infection in mice depresses contact sensitivity to oxazolone and enhances humoral response to several T-dependent and T-independent antigens (Campa *et al.*, 1975).

Since circulating antibodies are known to have suppressive effects on the cellular response against the same antigen (Uhr & Möller, 1968), it could be inferred that *P. aeruginosa* inhibits contact sensitivity by

TABLE 4. Effect of cyclophosphamide pretreatment on antibody response to SRBC in uninfected and infected mice

No. of mice	Cyclophosphamide pretreatment	Infection	PFC/10^6 spleen cells*	Haemcagglutinin titre†	Haemolysin titre†
15	—	—	648 (532–790)	8·5 (7·8–9·2)	8·5 (8·0–9·2)
15	—	0·2 LD_{50}	920‡ (787–1077)	9·5‡ (9·1–9·9)	9·7‡ (9·1–10·3)
13	20 mg/Kg	—	887 (635–1239)	9·1 (8·3–9·9)	9·3 (8·7–9·9)
13	20 mg/Kg	0·2 LD_{50}	978‡ (701–1362)	9·6‡ (8·8–10·4)	9·7‡ (9·0–10·4)
8	200 mg/Kg	—	21 (10–46)	0	0
8	200 mg/Kg	0·2 LD_{50}	27 (7–107)	2·6 (0·9–4·3)	2·1 (0·6–3·6)

* Data expressed as geometric mean; the 95% confidence limits are given in parentheses.

† Data expressed as arithmetic mean of $\log_2$ of the reciprocal values of titre; the 95% confidence limits are given in brackets.

‡ $P < 0{\cdot}02$, at least, when compared with non-pretreated uninfected controls.

eliciting a potentiated anti-oxazolone antibody response. However, we did not find significant changes in the anti-oxazolone titres when mice sensitized 24 hr after infection were challenged 6 days later.

It therefore seems unlikely that the major circulating classes of anti-oxazolone antibodies play a relevant role in the depression of contact sensitivity of *P. aeruginosa* infected mice. This interpretation is strengthened by the finding that contact sensitivity was already markedly depressed in mice sensitized 24 hr after infection and challenged 4 days later. In fact, there is evidence that at this time the B-cell response against oxazolone is not fully developed (Davies *et al.*, 1969; de Sousa & Parrot, 1969); thus, although direct measurements are not available, the anti-oxazolone antibody level may be considered low. However, the possibility that a minor antibody may be concerned cannot be excluded.

Moreover, such depression seems not to be due to impaired inflammatory response, which is important in the expression of contact sensitivity (Asherson & Barnes, 1973), since we found that this response is substantially unaffected by *P. aeruginosa* infection (Campa *et al.*, 1975).

Direct macrophage activation by *P. aeruginosa* could be the common pathway leading to enhanced humoral response and depressed contact sensitivity. In this regard, it has been shown that the systemic injection of killed *Corynebacterium parvum* can increase the phagocytic capability of spleen macrophages, which then amplify the B-cell response on the one hand, and deplete DTH-reactive T cells on the other, sequestrating, and possibly destroying them, in the spleen (Howard *et al.*, 1973; Scott, 1974).

We reported that, in mice inoculated with killed bacteria, the humoral response was potentiated, whereas contact sensitivity was not depressed (Campa *et al.*, 1975). It is conceivable in this respect that the requirements to activate macrophages for increased antibody production and depressed delayed hypersensitivity may be different. In particular, the effect on DTH might demand prolonged stimulation of macrophages, such as occurs during infection. However, recent work has shown that sublethal infection with *P. aeruginosa* in mice is characterised by a rapid decline in the number of bacilli during the first few hours after infection (Sensakovic & Bartell, 1974). Thus, the difference between killed and living bacteria might not be a quantitative one, but may derive mainly from the presence of distinct bacterial factors, which could then trigger different responses. In particular, enhanced antibody production to SRBC, observed both in mice inoculated with living bacteria and in mice injected with heat-killed micro-organisms, could well result from B-cell stimulation by a thermostable constituent of *P. aeruginosa*, such as the endotoxin (Campa *et al.*, 1975).

On the other hand, depression of contact sensitivity, observed only in infected mice, could be due to activation of suppressor cells.

Suppressor cells of different types can be produced by treatment with appropriate antigen and arise during conventional immunization. Asherson & Zembala (1974) found macrophages which can non-specifically inhibit the passive transfer of contact sensitivity to unrelated antigens. T-suppressor cells were produced in mice by the intravenous injection of picryl sulphonic acid, an agent which makes animals unresponsive to sensitization by picryl chloride (Zembala & Asherson, 1973). Phanuphak, Moorhead & Claman (1974) made similar observations using dinitrobenzene sulphonic acid and dinitrochlorobenzene. It has also been reported that there are B cells which suppress DTH in guinea-pigs sensitized with either ovalbumin in Freund's incomplete adjuvant or 2-4 dinitrofluorobenzene. Since the precursors of these cells were sensitive to CY, high doses of CY (200–300 mg/Kg) injected before sensitization cause an increase in contact sensitivity and a concomitant depression of antibody responses (Turk *et al.*, 1972; Turk & Parker, 1973; Polak & Turk, 1974; Katz *et al.*, 1974; Askenase *et al.*, 1975). These two effects of CY, however, are not causally related, since potentiation of delayed reactions is observed also with low doses of CY (20 mg/Kg) which do not depress, but sometimes enhance antibody responses (Askenase, Hayden & Gershon, 1975).

In our experiments, in fact, low doses of CY induced non-significant enhancement of humoral response to SRBC, whereas contact sensitivity was no longer depressed but potentiated, even though not significantly, in infected mice, and was normal in uninfected ones. When high doses of CY were used, humoral immunity was found to be completely depressed, whilst contact sensitivity was slightly increased. So, CY pretreatment at any dose level does not substantially alter contact sensitivity in uninfected mice, and prevents its depression in infected animals, no matter whether the antibody response is intact or suppressed. CY is known to affect preferentially, B rather than T lymphocytes (Turk *et al.*, 1972; Polak & Turk, 1974). Further, the recent work of Zembala & Asherson (1976) has shown that suppressor cells that inhibit the passive transfer of contact sensitivity in the mouse do not produce unresponsiveness, and stem from CY-sensitive precursors; on these grounds, the authors maintain that they are B cells. It might be inferred therefore that *P. aeruginosa* infection activates suppressor cells which have B-cell characteristics. Whether the effects of low doses of CY have an underlying mechanism common with the high doses remains, however, to be clarified.

The evidence here presented in favour of direct activation of suppressor cells in *P. aeruginosa* infected mice is clearly only inferential; the possibility that other mechanisms are operative in the contact sensitivity system of experimentally infected mice cannot be dismissed. Transfer studies would probably provide direct and definitive evidence on this point.

However, if the infection has an effect on suppressor cells, as indicated by these studies on contact sensitivity, then an effect should also be felt by the antibody response to heterologous erythrocytes, which normally requires cooperation between B and T lymphocytes. In fact, when normal mice were given low doses of CY, antibody production to SRBC was increased, but a further increment was also observed in the animals infected after CY administration.

In conclusion, *P. aeruginosa* infection: (i) stimulates B lymphocytes, either directly or by activation of reticuloendothelial cells in the spleen, and this effect may be related to the putative endotoxin; (ii) stimulates suppressor cells, which in turn depress contact sensitivity and modulate the B-cell response; this effect on suppressor cells is probably due to some *in vivo* released factor(s), since it occurs when living, but not killed bacteria, are injected.

The mechanisms of interaction between *P. aeruginosa* and suppressor cells need further investigation. Our data suggest that both the infection/sensitization order and the time interval between them are relevant. This is consistent with the idea that a bacterial factor has to be released. In addition, the antigen dose seems critical, suggesting that the interference of the infectious agent on the host's immune reactivity could include modulation of suppressor cell sensitivity to the antigen.

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