INFECTION AND IMMUNITY, Aug. 1978, p. 354-359
0019-9567/78/0021-0354$02.00/0

Vol. 21, No. 2
Printed in U.S.A.

Depression of Contact Sensitivity by Enhancement of Suppressor Cell Activity in *Pseudomonas aeruginosa*-Injected Mice

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Received for publication 16 March 1978

Heat-killed *Pseudomonas aeruginosa* depresses contact sensitivity to oxazolone in C56BL/6 mice. The draining lymph nodes and spleens of mice exhibiting an impaired reactivity to oxazolone contain a cell population capable of depressing the response to oxazolone of recipients sensitized immediately before cell transfer. The suppressive activity of these cells appears to be antigen specific, since they do not affect the response to picryl chloride and because they do not arise in *P. aeruginosa*-injected but not oxazolone-sensitized mice. These suppressor cells occur in the draining lymph nodes and spleen at 3 and 4 days after sensitization, respectively, and have precursors sensitive to cyclophosphamide. It is concluded that *P. aeruginosa* depresses contact sensitivity to oxazolone by enhancing the suppressor cell activity of the regulatory cells which arise during conventional sensitization.

Infections caused by *Pseudomonas aeruginosa* have become increasingly prevalent in hospitals. About 7 out of every 1,000 hospitalized patients develop *P. aeruginosa* infections (5). The use of antibiotics, immunosuppressive agents, and radiation therapy, as well as a considerable number of supportive procedures, increases the susceptibility of patients to infection by this microorganism. Although effective antibiotics are available, *P. aeruginosa* infection is a major cause of morbidity and mortality among patients with altered immune reactivity. Indeed, it is well known that there is a strikingly close relationship between life-threatening infection by this microorganism and such clinical conditions as severe burns, multiple trauma, and disseminated malignancies in which marked depression of cell-mediated immunity occurs. On the other hand, a number of previous studies (9, 19, 21), including investigations reported by us (6), have shown that *P. aeruginosa* itself affects cellular immunity in humans and laboratory animals. Furthermore, quite recently it has been suggested that cell-mediated immunity might be an important part of the normal host response to exposure to this microorganism (13). It is, therefore, critically important to understand the mechanism by which *P. aeruginosa* suppresses immune responsiveness, since the outcome of infection therapy may be intimately related to the status of the immune system. Our previous work (7) had suggested that the suppression of contact sensitivity observed in *P. aeruginosa*-infected mice might be due to the stimulation of suppressor cells. However, several questions remained to be answered regarding the immunosuppressive factor(s) of the microorganism and how it actually activates suppressor cells.

To assess whether the depression of contact sensitivity in *P. aeruginosa*-infected mice was due to some in vivo released factor(s) or to some constituent of the bacteria cell, in the investigation reported here mice were injected with heat-killed microorganisms. The results demonstrate that *P. aeruginosa*, even when administered as a heat-killed microorganism, inhibits contact sensitivity of mice sensitized with oxazolone by stimulating the suppressor cells which normally arise during sensitization. These cells, which have precursors sensitive to cyclophosphamide, appear to be antigen specific, since they fail to transfer the depression of contact sensitivity to recipient mice sensitized with a different sensitizing agent, picryl chloride.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 mice of both sexes raised in our institute and aged 8 to 12 weeks were used throughout this investigation. In each experiment the animals were randomly allocated to the different groups. Each group consisted of 7 or 8 mice.

Microorganism. A strain of *P. aeruginosa* isolated from clinical specimens was used in these experiments. A stock culture on Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.) was transferred to a synthetic medium (18) and incubated with shaking and aeration at 37°C. After 24 h, the cells were collected (10,000 × *g*, 15 min, 4°C), washed three times in distilled water, resuspended in it, killed by

heating at 65°C for 60 min, and then lyophilized.

Injection in mice. Mice were inoculated intravenously with (unless otherwise stated) 700 μg of heat-killed *P. aeruginosa* suspended in 0.5 ml of sterile saline immediately before injection. This suspension contained approximately 6×10^9 bacterial cells. In a control trial for acute toxicity, the injected mice were observed for 30 days, but no death occurred.

Sensitization. Mice were anesthetized by intraperitoneal administration of tribromoethanol in amylene hydrate (Avertin; Winthrop Laboratories, New York, N.Y.) and sensitized for contact sensitivity 24 h after *P. aeruginosa* injection by painting the skin of the abdomen and lower thorax with 0.2 ml of a 1% solution of 2-phenyl-4-ethoximethylene-oxazolone (oxazolone; British Drug Houses [BDH], Poole, England) or with 0.2 ml of a 3% solution of picryl chloride (BDH) in absolute ethanol.

Detection of contact sensitivity. Sensitized animals were challenged 6 days after sensitization by painting both sides of each ear with a drop of oxazolone or picryl chloride in olive oil. The quantification was made by measuring, with a micrometer, the increase in ear thickness 24 h later.

Cyclophosphamide treatment. Cyclophosphamide (Endoxan-Asta; Asta-Werke A.G.-Chemische Fabrik, Brackwede, W. Germany), dissolved in sterile saline immediately before use, was injected intraperitoneally as a single dose of 200 mg/kg (unless otherwise stated) 2 days before sensitization.

Lymph node cell transfer. Donor mice were injected with heat-killed *P. aeruginosa* and sensitized 24 h later with the chosen sensitizing agent on the abdominal skin. The donors were sacrificed 3 days after sensitization; the regional brachial and inguinal lymph nodes were removed and dissociated in Eagle minimal essential medium. The cells were washed twice, and viability was tested by trypan blue dye exclusion. Cell suspensions (0.5 ml) containing (unless otherwise stated) 50×10^6 viable cells were intravenously injected in recipient mice sensitized 1 h before with the contactant on the abdomen skin. Control groups consisted of sensitized mice receiving no cells.

The challenge of the recipients and controls was performed 6 days later as described above.

Spleen cell transfer. Donor mice were injected with heat-killed *P. aeruginosa* and sensitized 24 h later. The donors were sacrificed 4 days after sensitization; the spleens were removed, and from this point the procedure was as above.

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

RESULTS

Effect of heat-killed *P. aeruginosa* on contact sensitivity. Groups of normal mice and mice injected intravenously 24 h previously with varying doses of heat-killed microorganisms were sensitized with oxazolone. The challenge was performed with the same antigen 6 days later.

Contact sensitivity in injected animals was lower than that in controls at all tested doses, but it was markedly depressed ($P < 0.001$) only in the animals receiving 700 μg of bacteria (Fig. 1). This dose of heat-killed *P. aeruginosa*, injected 24 h before sensitization, was therefore used in all subsequent experiments.

The possibility that an altered reactivity of lymphocytes, as a direct toxic effect of the microorganism, might be responsible for the observed immunodepression should be excluded, since lymphocytes from mice injected with 700 μg of *P. aeruginosa* exhibited a normal mitotic response to mitogens (data not shown).

Effect of cyclophosphamide pretreatment on contact sensitivity to oxazolone in *P. aeruginosa*-injected mice. The cytotoxic drug cyclophosphamide has been extensively used to manipulate both humoral and cell-mediated immunity. In particular, enhanced delayed reactions result when cyclophosphamide is introduced before administration of the antigen, and this phenomenon appears to be due to the elimination of suppressor cells (10, 15).

An attempt to prevent the development of the depression of contact sensitivity in *P. aeruginosa*-injected mice was made with this drug. Four groups of mice were treated with 20, 50, 100, and 200 mg of cyclophosphamide per kg, respectively, and a fifth group was left untreated. A further five groups received the same doses of cyclophosphamide and were also injected with 700 μg of *P. aeruginosa* 24 h later. All 10 groups were sensitized with oxazolone 48 h after receiving the drug. Figure 2 shows that the *P. aeruginosa*-injected mice receiving 20 mg of cyclophosphamide per kg, as well as those receiving

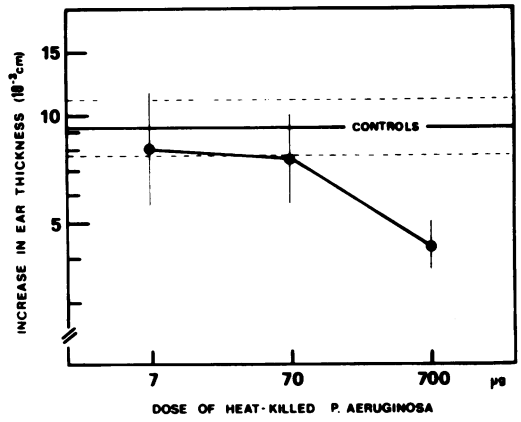

FIG. 1. *Effect of varying doses of heat-killed P. aeruginosa on contact sensitivity to oxazolone. Horizontal lines are the geometric mean and the 95% confidence limits of uninjected controls. Vertical bars indicate the 95% confidence limits of P. aeruginosa-injected animals.*

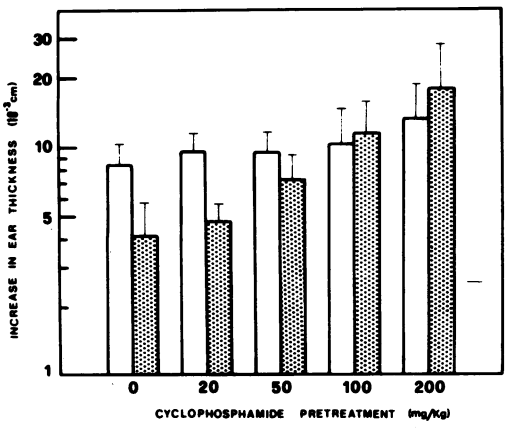

FIG. 2. *Effect of varying doses of cyclophosphamide on contact sensitivity to oxazolone in P. aeruginosa-injected (dotted columns) and uninjected (white columns) mice. Vertical bars indicate the upper 95% confidence limit. A positive linear relationship exists between the response to oxazolone of P. aeruginosa-injected mice and the dose of cyclophosphamide pretreatment (Y = 0.078 X + 3.385; r = 0.856; P < 0.001).*

no cyclophosphamide, exhibited a significant ($P < 0.001$) depression of contact sensitivity as compared with the respective control groups; when 50 mg of drug per kg was given, contact sensitivity was no longer found to be depressed. Furthermore, the ear swelling in the *P. aeruginosa*-injected mice receiving 100 mg, and even more in those given 200 mg, of cyclophosphamide per kg was actually greater than that of their uninjected littermates, although in neither case was the difference statistically significant. In fact, in the *P. aeruginosa*-injected mice a positive linear relationship between the dose of cyclophosphamide pretreatment and the response to oxazolone was found ($r = 0.856$; $P < 0.001$). In other words, not only does pretreatment of *P. aeruginosa*-injected mice with cyclophosphamide prevent the development of depression of contact sensitivity, but this effect seems to be dependent on the dose of drug administered. Also, in the case of the normal mice treated with cyclophosphamide it was observed that the response to oxazolone increased with increases in the dose of drug administered, but to a much less marked extent.

Contact sensitivity to oxazolone in mice receiving lymph node or spleen cells from *P. aeruginosa*-injected and oxazolone-sensitized donors. The above findings suggested that depression of contact sensitivity to oxazolone in *P. aeruginosa*-injected mice might be attributable to suppressor cells.

To test this hypothesis, recipient mice were sensitized with oxazolone and 1 h later were injected intravenously with 5×10^6 or 50×10^6 cells of brachial and inguinal lymph nodes from donors sensitized 3 days previously with the same antigen. This time interval between sensitization and cell transfer and this source of lymph node cells were chosen on the ground that it is known that in mice the passive transfer of contact sensitivity by draining lymph node cells peaks at day 3 after sensitization (3). One group of donors was also injected with *P. aeruginosa* 24 h before sensitization. The control group consisted of oxazolone-sensitized mice receiving no cells. The challenge of the experimental and control groups was performed with oxazolone 6 days after the cell transfer. The ear swelling of recipients inoculated with 5×10^6 lymph node cells from either *P. aeruginosa*-injected or uninjected donors was not significantly different from that of the control group. Also, 50×10^6 lymph node cells from uninjected donors did not alter the response to oxazolone in the recipients, whereas the same number of cells from injected donors induced a significant ($P < 0.01$) depression of contact sensitivity (Table 1, experiment 1). When spleen cells were used instead of lymph node cells, the donors were sensitized 4 days

TABLE 1. *Contact sensitivity to oxazolone in mice receiving lymph node or spleen cells from P. aeruginosa-injected and oxazolone-sensitized donors*

Expt no.[a]	Transferred cells ($\times 10^6$)	Contact sensitivity[b]
1	—	9.31 (6.06–14.30)
	5 LN (Ox)	8.46 (5.60–12.75)
	5 LN (PA Ox)	7.16 (4.94–10.37)
	50 LN (Ox)	11.44 (7.66–17.01)
	50 LN (PA Ox)	4.52[c] (3.16–6.46)
2	—	7.60 (6.40–9.03)
	50 SP (Ox)	7.27 (5.58–9.48)
	50 SP (PA Ox)	3.83[c] (3.06–4.79)

[a] —, None; LN, lymph node cells; SP, spleen cells; Ox, from oxazolone-sensitized animals; PA Ox, from *P. aeruginosa*-injected and oxazolone-sensitized animals.

[b] Expressed as increase in ear thickness 24 h after challenge in units of 10^{-3} cm. All data are expressed as geometric means; the 95% confidence limits are given in parentheses.

[c] Levels of significance when compared with the control group receiving no cells; experiment 1, $P < 0.01$; experiment 2, $P < 0.001$.

before the cell transfer because peak transfer of contact sensitivity by cells of this organ occurs at this time (3). As before, one group of donors was also injected with *P. aeruginosa* 24 h before sensitization, and sensitized mice receiving no cells were used as control. Again, it was found that 50×10^6 cells from uninjected donors did not affect contact sensitivity of the recipient mice, whereas the same number of cells from injected donors significantly depressed ($P < 0.001$) the response of the recipients to oxazolone (Table 1, experiment 2).

Specificity of the cells responsible for the depression of contact sensitivity to oxazolone in *P. aeruginosa*-injected mice. The above results clearly indicated the presence of suppressor cells in the draining lymph nodes and in the spleen, 3 and 4 days after sensitization, respectively, of sensitized mice injected with *P. aeruginosa*. This time interval after sensitization was used to investigate the specificity of the suppressor cells when cells from sensitized donors were transferred.

Normal mice, 1 h after sensitization with oxazolone or picryl chloride, were inoculated intravenously with 50×10^6 cells of brachial and inguinal lymph nodes from donors treated as described below and were challenged 6 days later with the same antigen used for sensitization. A further two groups of mice, receiving no cells but sensitized and challenged like the recipient mice, were used as controls. Cells from normal donors and those that had only been *P. aeruginosa* injected or only oxazolone sensitized did not alter the response to oxazolone in the oxazolone-sensitized recipients, whereas cells from donors that had been both *P. aeruginosa* injected and oxazolone sensitized were able to transfer depression of contact sensitivity to oxazolone to oxazolone-sensitized mice (Table 2). Furthermore, lymph node cells from mice that had been both *P. aeruginosa* injected and oxazolone sensitized failed to depress the response to picryl chloride of recipients sensitized with picryl chloride, and cells from donors that had been both *P. aeruginosa* injected and picryl chloride sensitized failed to depress contact sensitivity to oxazolone of recipient mice sensitized with oxazolone.

These results indicated that the cells responsible for the depression of contact sensitivity in *P. aeruginosa*-injected mice were antigen specific in that they required specific antigenic stimulation.

Effect of cyclophosphamide on the precursors of suppressor cells in *P. aeruginosa*-injected mice. Normal mice were sensitized with oxazolone and 1 h later were injected intravenously with 50×10^6 spleen cells from donors sensitized 4 days previously with the same antigen. Two groups of donors were also injected with either *P. aeruginosa* or 200 mg of cyclophosphamide per kg 24 or 48 h before sensitization, respectively. A third group of donors received both *P. aeruginosa* and cyclophosphamide. Sensitized mice receiving no cells were used as controls. The challenge of the experimental and control groups was performed with oxazolone 6 days after the cell transfer. Cyclophosphamide completely inhibited the development of suppressor activity in the spleens of mice injected with *P. aeruginosa* and sensitized with oxazolone (Table 3).

TABLE 2. *Specificity of cells which suppress contact sensitivity to oxazolone in P. aeruginosa-injected mice*

Transferred cells (50×10^6)[a]	Recipient sensitization	Contact sensitivity[b]
—	Ox	7.86 (6.01–10.30)
LN (−)	Ox	7.72 (5.51–10.84)
LN (PA)	Ox	7.05 (4.97–10.00)
LN (Ox)	Ox	8.35 (6.38–10.94)
LN (PA Ox)	Ox	3.71[c] (2.79–4.92)
—	PCl	7.62 (6.25–9.29)
LN (Ox)	PCl	6.84 (5.08–9.20)
LN (PA Ox)	PCl	6.41 (4.47–9.20)
LN (PCl)	Ox	7.63 (6.15–9.48)
LN (PA PCl)	Ox	7.58 (6.55–8.79)

[a] —, None; LN, lymph node cells; Ox, oxazolone, PCl, picryl chloride; −, from normal mice; PA, from *P. aeruginosa*-injected but not sensitized animals; Ox, from oxazolone-sensitized animals; PA Ox, from *P. aeruginosa*-injected and oxazolone-sensitized animals; PCl, from picryl chloride-sensitized animals; PA PCl, from *P. aeruginosa*-injected and picryl chloride-sensitized animals.

[b] See Table 1, footnote *b*.

[c] Level of significance when compared with the control group: $P < 0.001$.

DISCUSSION

The results show that heat-killed *P. aeruginosa* depresses contact sensitivity to oxazolone in C57BL/6 mice when injected intravenously 24 h before sensitization. The spleens and the draining lymph nodes of mice exhibiting an impaired reactivity to oxazolone contain a cell population capable of passively transferring the suppression of contact sensitivity to recipients sen-

TABLE 3. *Contact sensitivity to oxazolone in mice receiving 50 × 10^6 spleen cells from cyclophosphamide-pretreated, P. aeruginosa-injected, and oxazolone-sensitized donors*

Transferred cells[a]	Contact sensitivity[b]
—	8.00 (5.98–10.69)
SP (Ox)	9.76 (7.46–12.79)
SP (PA Ox)	4.95[c] (3.80–6.46)
SP (CY Ox)	10.48 (7.76–14.12)
SP (CY PA Ox)	9.78 (7.76–12.44)

[a] —, None; SP, spleen cells; Ox, from oxazolone-sensitized animals; PA Ox, from *P. aeruginosa*-injected and oxazolone-sensitized animals; CY Ox, from cyclophosphamide-pretreated and oxazolone-sensitized animals; CY PA Ox, from cyclophosphamide-pretreated, *P. aeruginosa*-injected, and oxazolone-sensitized animals.

[b] See Table 1, footnote *b*.

[c] Level of significance when compared with the control group: $P < 0.02$.

sitized immediately before the cell transfer with the same antigen. The suppressive activity of these cells appears to be antigen specific, since they do not affect the response to a different sensitizing agent, picryl chloride, and because they arise in *P. aeruginosa*-injected mice only when they are sensitized. These suppressor cells, which occur in the draining lymph nodes and spleen at 3 and 4 days after sensitization, respectively, have precursors sensitive to cyclophosphamide.

The impairment of immune response induced by certain microorganisms or microbial products is well known (16), but only recently have data about suppressor cells in some of these systems been reported. It has, in fact, been found that suppressor cells, lying in the T and adherent cell compartment of the spleen, occur during experimental infection with *Trypanosoma brucei* (8); furthermore, immunoglobulin-bearing cells from mice injected with an acid polysaccharide produced by *Serratia piscatorum* may have a role in the suppressive activity of the spleen (11), and spleens activated by lipopolysaccharide contain cells which suppress the response of normal cells to the antigen sheep erythrocytes in vitro (14).

However, several questions remain to be answered regarding the nature, the mode of induction, and the mode of action of suppressor cells induced by *P. aeruginosa* in oxazolone-sensitized animals. Asherson and co-workers have shown that the draining lymph nodes and spleens of mice painted on the skin with a contact-sensitizing agent contain: (i) B suppressor cells that appear from day 6 after sensitization onwards and which act on the effector stage of contact sensitivity (22), (ii) B suppressor cells which may be identical and which depress the induction of contact sensitivity (1), and (iii) T suppressor cells which depress the in vivo DNA synthesis response to the contactant (2). Moreover, these three kinds of cells arise during conventional immunization as regulatory cells and have precursors sensitive to cyclophosphamide (1, 2, 10). It has also been shown that macrophages can nonspecifically inhibit the passive transfer of contact sensitivity (4).

The specificity of suppression observed in our experiments, therefore, suggests that it is unlikely that macrophages are the suppressor cells in our model, although we have no direct evidence of this and the role of these cells needs further investigation. The sensitivity to cyclophosphamide of the cells responsible for the depression of contact sensitivity to oxazolone in *P. aeruginosa*-injected mice supports the hypothesis that they may be B cells, since it is known that cyclophosphamide affects B rather than T lymphocytes (15, 17, 20). Furthermore, their early presence in the draining lymph nodes and spleen (within 3 and 4 days after sensitization, respectively) and their ability to depress the response to oxazolone of normal recipients when transferred at the time of recipient sensitization suggest that these cells could be the B suppressor cells described by Asherson as depressing the induction of contact sensitivity (1).

However, at the present state of knowledge, the T suppressor cells, described by several authors (2, 12), which limit the in vivo DNA synthesis response of lymphocytes to antigen stimulation and which are also sensitive to cyclophosphamide cannot be excluded either. Further experiments are being undertaken to determine the type and the mechanism of action of suppressor cells involved in this model.

In conclusion, the results reported here corroborate the findings of our earlier studies and clearly show that *P. aeruginosa* depresses contact sensitivity to oxazolone by enhancing the suppressor activity of the regulatory cells which arise prematurely during immunization, although we have not yet identified the bacterial factor responsible for this phenomenon.

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