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The Influence of Cyclophosphamide on Accumulation of Peritoneal Exudate Cells Responding to PPD in BCG-sensitized Guinea Pigs

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ABSTRACT. In BCG-sensitized guinea pigs, the influence of pretreatment with cyclophosphamide (CY) (300 mg/kg) 3 days before sensitization on peritoneal exudate cell accumulation in the PPD-induced delayed type hypersensitivity reaction was analyzed by using the sponge implant method. The number of polymorphonuclear neutrophils (PMN) accumulating in the intraperitoneally implanted sponges containing 50 μg of PPD was increased more than that in the untreated group 24-48 hours after challenged implantation when CY pretreated. The accumulation of mononuclear cells, however, was decreased. The enhanced effect of CY pretreatment on the immune response to PPD in tuberculin type of delayed type hypersensitivity through the selective inhibition of suppressor cells may be evaluated in terms of the increase in PMN accumulating at the reaction site.

Key words : Tuberculin reactions — Cyclophosphamide — Peritoneal exudate cells — Sponge implantation

Guinea pigs injected intraperitoneally with cyclophosphamide (CY) (300 mg/kg) 3 days before contact sensitization with 2,4-dinitrofluorobenzene or oxazolone showed markedly increased contact sensitivity reactions when skin tested 7 days later.[1,2] A similar increase in 24, 48 and 72 hours reactions were found in animals sensitized with ovalubumin (OA) in Freund's incomplete adjuvant after pretreatment with CY 3 days before sensitization.[3] However, it has been shown that the CY pretreatment did not increase reactivity to purified protein derivatives of tuberculin (PPD) in guinea pigs sensitized with BCG vaccine 7 days previously nor to OA in animals sensitized with OA in Freund's complete adjuvant (FCA).[2,4]

On the other hand, the sponge implant method was performed on BCG-sensitized guinea pigs by implanting PPD-impregnated gelatin sponges (PPD-sponge) into their peritoneal cavity. Delayed type hypersensitivity reactions were incited by the impregnated sponges in the peritoneal cavity that was the designated reaction site *in vivo*. The intraperitoneal implantation of such a spongy biomaterial impregnated with an antigen induces the accumulation of peritoneal exudate cells responding to antigen within the implanted sponge in sensitized animals.[5-7]

The influence of CY pretreatment on peritoneal exudate cell accumulation in the PPD-induced delayed type hypersensitivity reaction was analyzed by using

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this method, which provides quantitative collection and population analysis of peritoneal exudate cells that accumulate within the implanted PPD-sponges in guinea pigs sensitized with BCG in FCA.

MATERIALS AND METHODS

Animals. Female English Hartley guinea pigs weighing between 300–450 g were used throughout these investigations.

Immunization. Guinea pigs were sensitized by injecting into the foot pads 2.5 mg BCG (Kyowa Chemical Industries Ltd.) emulsified in Freund's complete adjuvant (Difco Lab.). They were challenged 12 days later by implanting gelatin sponges ($1 \times 1 \times 2.5$ cm Yamanouchi Pharmaceuticals Co.) containing 5 μg or 50 μg of PPD (Kainosu Co.) into the peritoneal cavity.

Treatment of guinea pigs. Thirty guinea pigs were investigated per group and 6 guinea pigs per subgroup or time point. All guinea pigs were given CY (300 mg/kg, Shionogi Pharmaceuticals Co.) intraperitoneally or no CY.

Histological examination. The guinea pigs were killed 24, 48 or 72 hours after PPD-sponge implantation. The PPD-sponges were immediately removed, fixed in 10% neutral formalin, and embedded in paraffin. Serial sections 6 to 8 μm thick were stained with Hematoxylin and Eosin for morphologic examination. Five sections per sponge were measured and the number of cell per 1 ($=0.0625 \times 16$) mm^2 of sections was counted.[8] The mean of the cell counts for 6 individual sponges was given.

RESULTS

Accumulation of peritoneal exudate cells in the PPD-sponges.

In both BCG-sensitized and non-sensitized animals, the peritoneal exudate cells that had accumulated in the implanted PPD-sponges differentiated into mononuclear (Mono) cells (MN) and polymorphonuclear (Poly) neutrophils (PMN) (Table 1). In the sensitized animals, the number of MN accumulating in the sponges containing 50 μg of PPD was 20–25% greater than that in the sponges containing 5 μg of PPD 48 and 72 hours after challenged implantation. This indicated that the PPD doses affected the accumulation of MN populations in the tuberculin type of delayed type hypersensitivity reactions. The accumulation of PMN, however, was unaffected.

Effect of CY on tuberculin responsiveness.

In the sensitized animals, the number of MN accumulating in the sponges containing 50 μg of PPD was smaller than that in the untreated group 24–72 hours after challenged implantation when CY was administered 3 days before or 1 day after the sensitization (Table 2). On the other hand, the MN accumulation in the sponges containing 5 μg of PPD was comparable to that of the untreated group 24 hours after implantation but smaller after 48–72 hours when CY was administered 3 days before or 1 day after the sensitization. Accumulated MN populations in the sponges containing 50 or 5 μg of PPD are not only included subpopulations that are dose-dependently responsive to challenged antigen, but also each subset that is indirectly or specifically affected by the

TABLE 1. Numerical density of accumulated exudate cells in the intraperitoneally implanted gelatin sponges in BCG-sensitized and non-sensitized guinea pigs.

Group		Exudate cell count analysis		
		Time after implantation		
		24h	48h	72h
Sensitized animals				
1. PPD-sponge implants (5 μg^a)	Mono	993± 79	1253±65	1234±83
	Poly	1715±103	830±77	471±44
2. PPD-sponge implants (50 μg^b)	Mono	963± 90	1539±83*	1650±96**
	Poly	1802±109	756±72	431±58
Non-sensitized animals				
3. PPD-sponge implants (50 μg)	Mono	612± 59**	898±66**	933±62*
	Poly	835± 88***	314±45***	174±17***

Each group consisted of 6 guinea pigs. Each guinea pig was intraperitoneally implanted a gelatin sponge containing 5 μg^a or 50 μg^b PPD. Results were expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges ±SEM. *$p<0.01$, **$p<0.005$, ***$p<0.001$ versus PPD-sponge implants (5 μg) group.

TABLE 2. Effect of CY on the accumulation of exudate cells in the intraperitoneally implanted gelatin sponges in BCG-sensitized guinea pigs.

Group/treatment		Exudate cell count analysis		
		Time after implantation		
		24h	48h	72h
Sensitized animals				
1. No treated				
PPD-sponge implants (50 μg^a)	Mono	963± 90	1539±83	1650±96
	Poly	1802±109	756±72	431±58
2. ^{b}CY treated				
PPD-sponge implants (50 μg)	Mono	658± 56*	1248±77*	1332±81*
	Poly	2195±131*	1170±75*	579±43
3. ^{c}CY treated				
PPD-sponge implants (50 μg)	Mono	553± 54**	1223±71*	1286±75*
	Poly	1000± 76***	503±63*	149±20**
4. ^{d}CY treated				
PPD-sponge implants (50 μg)	Mono	114± 14***	143±30***	154±18***
	Poly	1711± 70	818±60	392±28
5. ^{e}CY treated				
PPD-sponge implants (50 μg)	Mono	138± 15***	174±17***	362±47***
	Poly	117± 29***	41±13***	10± 3***

Each group consisted of 6 guinea pigs. Each guinea pig was intraperitoneally implanted a gelatin sponge containing 50 μg^a PPD: ^{b}CY (300 mg/kg) was administered intraperitoneally 3 days before sensitization. ^{c}CY (300 mg/kg) was administered intraperitoneally 1 day after sensitization. ^{d}CY (300 mg/kg) was administered intraperitoneally 1 day before challenge. ^{e}CY (300 mg/kg) was administered intraperitoneally 3 days before challenge. Results were expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges±SEM. *$p<0.01$, **$p<0.005$, ***$p<0.001$ versus no treated group.

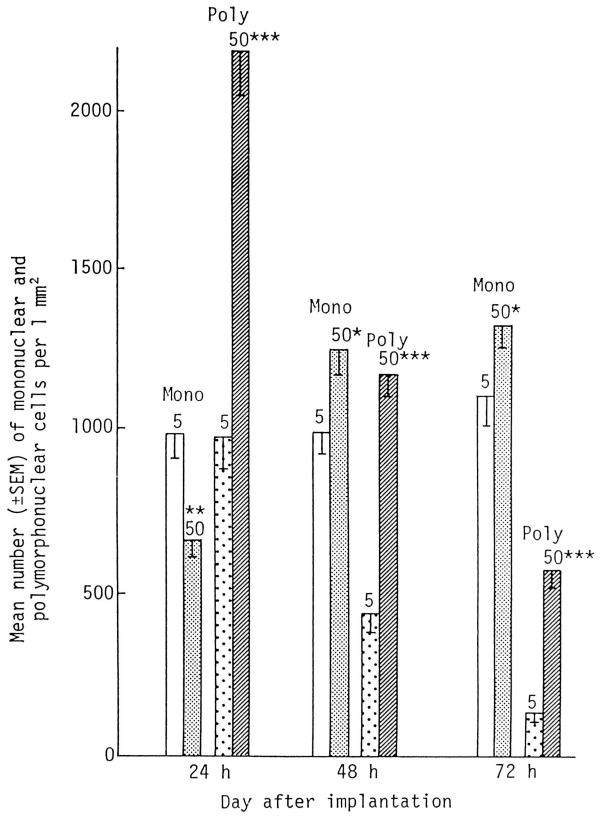

Fig. 1. Effect of CY on the accumulation of mononuclear cells and polymorphonuclear neutrophils in the intraperitoneally implanted gelatin sponges containing 5 μg (5) or 50 μg (50) PPD in BCG-sensitized guinea pigs. Guinea pigs were administered intraperitoneally CY (300 mg/kg), 3 days before sensitization. Mono in sponges containing 5 μg PPD (□) and in sponges containing 50 μg PPD (▨). Poly in sponges containing 5 μg PPD (⁘) and in sponges containing 50 μg PPD (▨). Results were expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges$\pm$SEM. *$p<0.01$, **$p<0.005$, ***$p<0.001$ versus PPD sponge implants (5 μg) group.

treatment with CY 3 days before and 1 day after the sensitization (Figs. 1 and 2).

PMN accumulating in the sponges containing 50 μg of PPD was increased compared to that of the untreated group 24-48 hours after challenged implantation, only when CY was administered 3 days before the sensitization (Table 2), but the cells in the sponges containing 5 μg of PPD decreased between 24-72 hours after challenged implantation.

Effect of CY on non-specific inflammation to the PPD-sponges.

In the non-sensitized animals, MN and PMN accumulating in the implanted PPD-sponges, which acted as inflammatory agents, contained their respective CY-sensitive cell populations. The difference in the sensitivity of these subpopulations to CY was dependent on the interval between the CY administration and the sponge implantation (Table 3). However, a CY administration

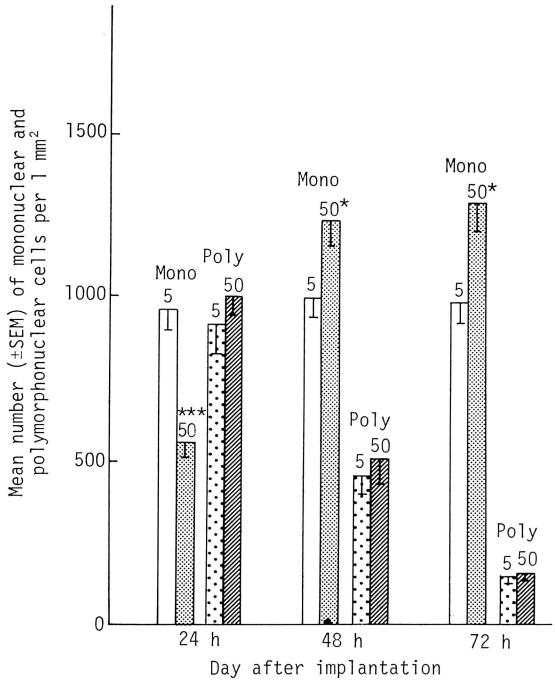

Fig. 2. Effect of CY on the accumulation of mononuclear cells and polymorphonuclear neutrophils in the intraperitoneally implanted gelatin sponges containing 5 μg (5) or 50 μg (50) PPD in BCG-sensitized guinea pigs. Guinea pigs were administered intraperitoneally CY (300 mg/kg), 1 day after sensitization. Mono in sponges containing 5 μg PPD (□) and in sponge containing 50 μg PPD (▨). Poly in sponges containing 5 μg PPD (⬚) and in sponges containing 50 μg PPD (▨). Results were expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges$\pm$SEM. *$p<0.01$, ***$p<0.001$ versus PPD-sponge implants (5 μg) group.

12 days before the sponge implantation did not affect the accumulation of MN or PMN in the PPD-sponges.

DISCUSSION

In the tuberculin type of delayed type hypersensitivity, the development of skin reactivity is usually evaluated by skin tests consisting of such items as erythema, induration, and the diameter of the skin reaction site. Pretreatment with CY (300 mg/kg) 3 days before sensitization with 0.25 mg of BCG is reported to reduce skin reactivity when the magnitude of the tuberculin reaction is evaluated according to these indices.[2] However, in guinea pigs sensitized with 0.4 ml of M. tuberculosis H37Ra, which produces stronger delayed-type hypersensitivity reactions to PPD, CY pretreatment caused no significant changes in the tuberculin reactions test in terms of the diameter of the skin reactions but enhanced the induration.[9] Moreover, in these animals, histological examination of biopsy specimens obtained 24 hours after the skin test revealed that the degree of the marked edema resulting from the enhanced

TABLE 3. Effect of CY on the accumulation of exudate cells in the intraperitoneally implanted gelatin sponges in non-sensitized guinea pigs.

Group/treatment		Exudate cell count analysis		
		Time after implantation		
		24h	48h	72h
Non-sensitized animals				
1. No treated				
PPD-sponge implants (50 μg[a])	Mono	612±59	898±66	933±62
	Poly	835±88	314±45	114±17
2. [b]CY treated				
PPD-sponge implants (50 μg)	Mono	315±29**	323±34***	329±34***
	Poly	729±68	361±41	83±13
3. [c]CY treated				
PPD-sponge implants (50 μg)	Mono	326±35**	332±33***	328±29***
	Poly	90±26***	47± 9***	27±11***
4. [d]CY treated				
PPD-sponge implants (50 μg)	Mono	600±51	918±60	949±58
	Poly	941±71	308±40	144±24

Each group consisted of 6 guinea pigs. Each guinea pig was intraperitoneally implanted a gelatin sponge containing 50 μg[a] PPD. [b]CY (300 mg/kg) was administered intraperitoneally 1 day before implantation. [c]CY (300 mg/kg) was administered intraperitoneally 3 days before implantation. [d]CY (300 mg/kg) was administered intraperitoneally 12 days before implantation. Results were expressed as mean counts per 1 ($=0.0625\times16$) mm^2 of 30 sections from 6 sponges ±SEM. **$p<0.005$, ***$p<0.001$ versus no treated group.

PPD reactions by the CY pretreatment was directly correlated to the number of polymorphonuclear leucocytes present in the infiltrate.[9] Thus, we explored the possibility of evaluating this enhancement of delayed-type hypersensitivity by quantitative analysis of exudate cells accumulated in intraperitoneal sponges containing 5 μg or 50 μg of PPD in guinea pigs strongly sensitized with 2.5 mg of BCG in FCA. The CY pretreatment initiated a striking increase in the number of PMN and a decrease in MN in the sponges containing 50 μg of PPD during the 24 hours following the implantation. In the 5 μg PPD sponges, on the other hand, the number of PMN decreased but that of MN did not change during this period. The CY pretreatment is considered to affect delayed type hypersensitivity reactions by eliminating populations of suppressor cells[4,10] and, thus, changing the magnitude of exudate cell accumulation at the immune reaction site of peritoneally implanted sponges.

In the non-sensitized guinea pigs, administration of CY 12 days before the sponge implantation had no effect on the accumulation of MN and PMN. Therefore, the suppressive effect of CY administered 3 days before or 1 day after the sensitization on the accumulation of peritoneal exudate cells is not considered directly to have been influenced by beginning time of the sponge implantation. On the other hand, suppression of the proliferation of the CY-sensitive cell populations is also evident in tuberculin reactions. Our data indicate that the accumulated exudate cells at the immune reaction site of peritoneally implanted sponges consist not only of the non-specific infiltrated or the CY-non-sensitive cell populations but also the specific infiltrated or the CY-sensitive cell populations that influence the magnitude of the

immune response against PPD.

In the sensitized guinea pigs, while the total number of peritoneal exudate cells accumulating in the sponges containing 5 μg of PPD was smaller in the CY pretreated group than in the untreated group, the total number in the CY pretreated group with 50 μg PPD sponges was comparable to that of the untreated group due to the increase in PMN which compensated for the decrease in MN during the 24–72 hour period (Table 2). This enhanced accumulation of PMN may be symptomatically equivalent to the marked edema and the resultant increase in induration in response to 25 μg of PPD in M. tuberculosis H37Ra-sensitized guinea pigs.[9] Therefore, the enhanced effect of CY on tuberculin reactions through the selective inhibition of suppressor cells may be evaluated in terms of the increase in PMN accumulating at the immune reaction site.

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