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Effects of Cyclophosphamide on Susceptibility to Experimental Infection of Mice and Rabbits with *Pasteurella multocida*

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Introduction

The cytotoxic drug cyclophosphamide (CY) has been used extensively as an immunosuppressant, since it was first synthesized by Arnold, Bourseaux and Brok.[5,7] CY is an alkylating agent whose cytotoxic action is directed at actively dividing cells. The effects of CY on immune responsiveness has been evaluated in a variety of animals, such as mice[15,25], guinea-pigs[1], rats[21] and domestic animals[7,16], and was reported that CY was toxic to B-cells to reduce the humoral immune response and depletes non-thymus dependent areas of lymph nodes and spleens in guinea-pigs[17,22] mice[22] and calves.[7] It has also been reported that different doses and schedules of CY administration result in different effects on humoral and cell-mediated immunity[5,13,25].

CY-induced immunosuppression was recently used in pathogenic investigations of bacterial and viral infections in laboratory animals and livestock.[7,18] It has been well documented that experimental infection of bacterial organisms and viruses to the respiratory system is hard to make success in most species of mammals, as the respiratory tracts are well installed with immunobiological defence mechanisms.[6,12,14,24]

The use of CY is of interest to evaluate relationships between host immune mechanisms and susceptibility to pulmonary infection of mice and rabbits with *Pasteurella multocida*. The present report describes the results of investigations on the effects of CY on susceptibility to experimental infection in the respiratory tracts of mice and rabbits with a strain of *P. multocida*, that was isolated from a rabbit with enzootic pasteurellosis (snuffles).

Materials and Methods

Animals: The disease-free ICR male mice, weighing 16 to 18 g and bred in the Institute of Veterinary Research, were used. New Zealand White male rabbits weighing 2 to 2.5 kg were obtained from the healthy colonies in the rabbitry equipped with battery cages. The rabbits were clinically observed for 2 weeks before the start of experiments to confirm healthy conditions. All animals were housed in an environment in which there was a minimal chance of exposure to pathogenic micro-organisms.

Cyclophosphamide Treatment: Cyclophosphamide (Endoxanasta, Asta-werke, Germany; CY) was prepared as recommended by the manufacturer and administered intraperitoneally (i.p.) to mice with a dose of 200mg CY per kg body weight, and intravenously (i.v.) to rabbits with a dose of 150mg CY per kg body weight. The controls (the non-CY-treated) were injected 0.85% saline alone by the same manner or CY injection.

Intranasal Inoculation: A strain of *P. multocida* (PMC-1) of capsular serogroup A isolated from a rabbit lung affected with snuffles was used. At the second passage in Bacto-tryptose phosphate broth, the viable cell counts of the bacteria were made by the use of Bacto-tryptose agar. The bacterial suspension at the concentration of 1×10^8 cells/ml was inoculated intranasally (i.n.) to mice and rabbits with the volumes of 0.1ml and 0.5ml, respectively. The controls were given 0.85% saline in the same ways of PMC-1 inoculation.

Recovery of Bacteria: Bacto-tryptose agar supplemented with 10% of sheep blood, Bacto-MacConkey agar and Bacto-tryptose broth were employed for bacterial isolation from the respiratory organs of

the experimental animals. Biochemical characterization of the isolates was carried out by the methods described by Cowan.[8] To identify the capsular serogroups of *P. multocida* isolated from the experim ental animals, the indirect hemagglutination test (IHA) described by Carter[4] was applied as mentioned below. The standard *P. multocida* strains, TS 8 (serogroup A), R 473 (serogroup B), P 27(serogroup D) and Bunia II (serogroup E), and hyper-immune sera of the strains prepared in rabbits were kindly provided by the Division of Bacteriology in the Institute. It was considered as recovery of PMC-1 if the isolated strains revealed the morphology and biochemical properties of *P. multocida*, and were identified with capsular serogroup A.

Antibody response: Cater's[4] indirect hemagglutination test (IHA) was employed with slight modification to measure the antibody titers of the sera from the experimental animals against PMC-1 antigen. With plastic hemagglutination macroplates, 0.4ml system by adding 0.2ml of diluted sera and 0.2ml of PMC-1 sensitised type 0 human erythrocytes was employed.

Hematological Study: Blood samples were collected weekly from the ear veins of rabbits in the bottles with double oxalate mixture as anticoagulants.[10] Total numbers of leucocytes were measured by means of hemacytometer, and differential counts of leucoytes were made using Giemsa stain.[10]

Histopathological Study: The experimental animals were autopsied within the observation periods or at the termination of experiments. The respiratory organs and spleens were taken at autopsy, fixed in 10% formalin and embedded in paraffin, followed by hematoxylin and eosin staining.

Results

Effects of CY Treatment to Mice: To test the effects of CY on susceptibility to experimental infection of mice with *P. multocida* (PMC-1), 12 mice were allotted in each of 3 groups; CY-treated, non-CY-treated and control (Table 1). PMC-1 was inoculated to the CY-treated and to the non-CY-treated at one week post inoculation (p.i.) of CY. The controls were inoculated i.n. with saline instead of PMC-1. As results summarised in Table 1, prominantly increasing mortality was observed in the CY-treated during the 14 days observation after PMC-1 incoulation, whereas none of the non-CY-treated and controls was died. At gross and microscopic examinations, the CY-treated revealed higher incidence of pneumonia, and severe atrophy of spleen with evident depletion of lymphocytes in the periarteriolar regions and red pulps accompanied hemorrhages. The degenerative changes of spleen were most apparent in the specimens taken from the CY-treated died at 3 to 6 days p.i. of PMC-1. No retrogressive changes were observed in the spleens from the non-CY-treated and controls. The trials on recovery of PMC-1 from lungs resulted in 66.7% of recovery in the CY-treated, and 25% in the non-CY-treated, while the controls showed no recovery of PMC-1.

Table 1. Effects of Cyclophosphamide (CY) on Susceptibility to Experimental Infection of Mice with *Pasteurella multocida* (PMC-1)

Groups	No. of Animals	Intranasal Infection	Mortality+	Pneumonia+	Recovery of PMC-1
CY-treated*	12	0.ml PMC-1**	5/12 (41.7)	9/12 (75.0)	8/12 (66.7)
Non-CY-Treated	12	0.1ml PMC-1	0/12 (0)	3/12 (25.0)	3/12 (25.0)
Control	12	0.1ml Saline	0/12 (0)	0/12 (0)	0/12 (0)

* Mice were injected i.p. with 200mg/kg body weight.

** *P. multocida* (PMC-1) at concentration of 1×10^7 cells in 0.1ml was inoculated i.n. at one week p.i. of CY.

+ Mortality and incidence of pneumonia were recorded during the 14 days observation after i.n. PMC-1 infection.

Numbers in parentheses indicate percentages of the groups.

Effects of CY Treatment to Rabbits

a) Spontaneous Induction of Snuffles: Following a single injection of CY to 12 rabbits and of saline to 8 rabbits, the animals were bred under the same conditions and clinically observed for 6 weeks. As results shown in Table 2, snuffles were appeared in 2 rabbits of the CY-treated at 3 to 4 weeks p.i. of CY, while none of the controls revealed the sym ptoms during the 6 weeks observation. At necropsy and microscopic examinations of the 2 rabbits, pneumonitis were observed mainly in the apical and diaphragmatic lobes. The spleens from the rabbits showed moderate atrophy and depletion of the cells in the periarteriolar regions and red pulps accompanied the hemosiderin. Bacterial examination of the affected lungs revealed that the penumonitis were associated with *Staphylococcus* spp., *E. coli* and *P. multocida* (serogroup D). The numbers of total leucocytes and mononucleated cells in the CY-treated decreased significantly ($P<0.05$) between 1 to 3 weeks p.i. of CY as compared with those of the controls, showing the lowest value at the 2nd weeks p.i. of CY (Fig.1).

Table 2. Effects of Cyclophosphamide (CY) on Spontaneous Induction of Snuffles in Rabbits

Groups	No. of Animals	Incidence of Snuffles at Weeks p.i.						Incidence Rate (%)
		1	2	3	4	5	6	
CY-Treated*	12	0	0	1	1	0	0	2/12(16.7)
Control	8	0	0	0	0	0	0	0/8 (0)

* Rabbits were inoculated i.v. with 150mg/kg body weight.

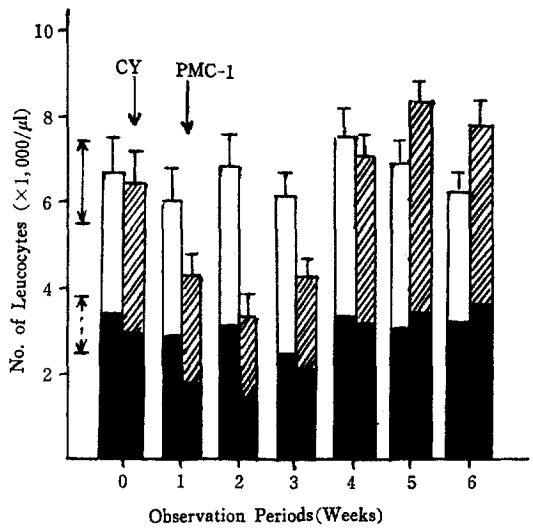

Fig 1. Effects of cyclophosphamide (CY) on total and differential leucocyte counts in rabbits. □■; the mean value±SE of 5 normal rabbits. ▨■; the mean value±SE of 5 CY-treated rabbits. Black bars indicate the mean numbers of mononucleated cells in the groups. Rabbits were injected i.v. with 150mg CY per Kg body weight.

b) Experimental Infection to Rabbits: Remaining 18 rabbits from the preceeding expeeriments were used for the experiment, following the 5 weeks clinical observation and confirmation of healthy condition. Ten rabbits were grouped for the CY-treated, 5 for the non-CY-treated and 3 for the control. PMC-1 was inoculated to the CY-treated and the non-CY-treated at one week p.i. of CY, and saline to the controls (Table 3). The results are presented in Table 3. During the 6 weeks observation, incidence of snuffles and pneumonia increased prominantly in the CY-treated as compared with the non-CY-treated and the controls. In the histopathological study conducted at the end of experiment, there was no difference in cellular populations appearing in the pneumonia between the CY-treated and the non-CY-treated. The spleens of CY-treated showed slight increase in size, but no histopathological changes in the parenchyma, whereas those of the non-CY-treated were observed with moderate cellular hyperplasia in the white and red pulps. Recovery rate of PMC-1 from lungs was much higher (60%) in the CY-treated than in the non-CY-treated (20%). Hematological studies revealed that the numbers of total leucocytes and mononucleated cells decreased transiently in the CY-treated at 1 to 3 weeks p.i. of CY (Fig. 2). At 5 to 6 weeks the total numbers of leucocytes of the CY-treated increased higher ($P<$

Table 3. Effects of Cyclophosphamide (CY) on Susceptibility to Experimental Infection of Rabbits with *Pasteurella multocida* (PMC-1)

Groups	No. of Animals	Intranasal Inoculation	Incidences of+		Recovery of PMC-1
			Snuffles	Pneumonia	
CY-Treated*	10	0.5ml PMC-1**	8(80)	9(90)	6/10(60)
Non-CY-Treated	5	0.5ml PMC-1	2(40)	2(40)	1/5 (20)
Control	3	0.5ml Saline	0(0)	0(0)	0/3 (0)

* Rabbits were injected i.v. with 150mg/kg body weight.

** *P. multocida* (PMC-1) at concentration of 5×10^7 cells in 0.5ml was inoculated i.n. at one week p.i. of CY.

+ Incidences of snuffles and pneumonia were recorded by clinical observation and histopathological reading of the respiratory organs from the rabbits sacrificed at the end of the 6 weeks observation. Numbers in parentheses indicate percentages of the groups.

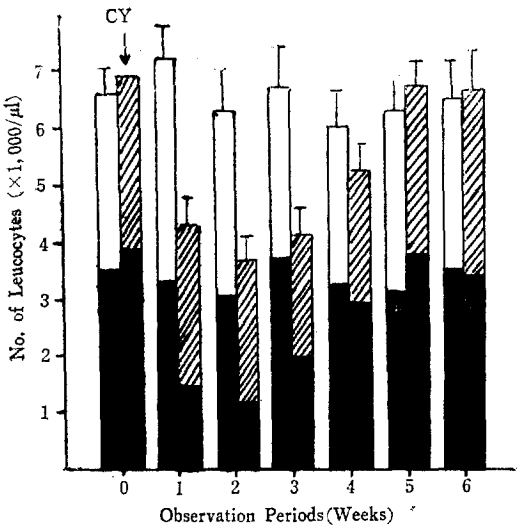

Fig 2. Effects of cyclophosphamide (CY) on total and differential leucocyte counts in association with i.n. experimental infection of rabbits with *Pasteurella multocida* (PMC-1).
□■: the mean value±SE of the non-CY-treated group with 5 rabbits inoculated with PMC-1 at one week p.i. of 0.85% saline.
▨■: the mean value±SE of the CY-treated group with 5 rabbits inoculated with PMC-1 at one week p.i. of CY.
Black bars indicate the mean numbers of mononucleated cells in the groups. The signs ⟷ and ⟷ indicate the ranges of No. of total leucocytes and mononucleated cells of 3 rabbits in the normal control group during the 6 weeks observation, respectively.

0.05) than those of the non-CY-trated, while no difference in the numbers of mononucleated cells between the CY-treated and the non-CY-treated was observed at the period.

c) Antibody Response: As the results shown in Fig. 3, the CY-treated group showed delayed and lower antibody responses against PMC-1 antigen for the overall 6 weeks observation period, as compared with those of the non-CY-treated. No significant changes of the antibody titers were recognized in the normal controls for the period.

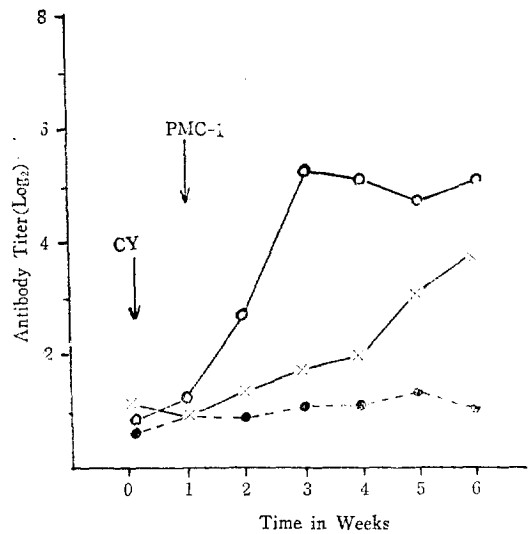

Fig 3. Changes in the mean antibody titer measured by indirect hemagglutination test (IHA) against homologous antigen following experimental infection of rabbits with *Pasteurella multocida* (PMC-1). PMC-1 was inoculated i.n. to 5 cyclophosphamide (CY)-treated (×) and non-CY-treated (o). ●;3 normal controls.

Discussion

Pasteurella multocida, a gram-negative bacterial parasite, has been considered as one of the major causative agents for respiratory diseases (snuffles) of domestic rabbits.[11,19] It has been suggested that the clinical symptoms of the pneumonia induced by *P. multocida* or other organisms are aggregated by pregnancy, parturition, heavy lactation and poor environmental conditions, probably associated with depression of general host immunity.[19] However, there is no evident consensus with regard to that host immunity, either systemic humoral or cellular defense mechanisms, is related to susceptibility to such microbial pathogens infected through the respiratory tracts of rabbits.

The present study has utilized cyclophosphamide (CY), an immunosuppressant, to induce depression of host immunity, and attempted to define the relationships between host immunity and susceptibility of pulmonary infection of *P. multocida* in mice and rabbits. Previous studies have established that a single injection of CY at dose of 300mg/kg selectively depletes non-thymus dependent areas of lymph nodes and spleens in guinea-pigs[17,22] and mice.[22] In addition, the B-cell population was almost completely eliminated from peripheral blood and lymphoid organs, while T-cell population partly affected by CY treatment.[1,7,13,25] Recently it has been reported that immunosuppressed hosts following treatment of CY,[20] glucocorticosteroids,[2] irradiation[6,23] or viral infection[3,9,14,26] have depression of normal pulmonary host defeses against *Pseudomonas aeruginosa*,[20] *Pneumococcus*[9,14] and *Hemophilus influenzae*,[9] and manifest increasing susceptibility to intra-pulmonary infection of such organisms.

The present results demonstrate that immunosuppression induced by CY treatment results in higher susceptibility to intranasal infection of rabbits and mice with *P. multocida*. Moreover, the provided evidences indicate that the increasing susceptibility is due to decreasing systemic as well as local immunodepression of the hosts treated with CY is proved with decreasing numbers of mononucleated cells in peripheral blood (Figs. 1&2) and depressing antibody responsiveness of the CY-treated groups during the periods of leucopenia(Fig. 3). Furthermore, atrophic spleens accompanied depletion of lymphocytes in the periarteriolar regions and red pulps provide the evidences that the host immune systems are effectively suppressed by the doses of CY treated. It has been generally known that the decreasing numbers of circulating mononucleated cells and bone marrow precurs are closely correlated with host immunosuppression and induction of higher susceptibility to microbial agents.[1,7,23] Similar changes of spleen to the present results have been observed in the mice,[22] guinea-pigs[17,22] and cattle[7] treated with CY. The present findings particularly corresponds with the observations of Corrier *et al.*[7] that the lymphocyte population in the cattle treated with CY was depleted in the cortex of the thymus and B-dependent areas of spleen and lymph nodes, and that antibody responsiveness to bacterial injection was delayed and diminished. Additionally, spontaneous occurrence of snuffles in 2 rabbits (Table 2) offers further evidence of the effect of CY-induced immunosuppression on the host's defense mechanisms, although the effects of CY on spleen and immune response were transient.

In guinea-pig, histologically complete restoration of lymphoid tissues following intraperitoneal inoculation of a dose of 280mg CY/kg occurred at 20 days p.i. of CY.[17] However, in rabbit, the depleted changes of the spleen in the CY-treated were evident at 3rd week p.i. of CY, but the spleen at 6th week p.i. of CY showed histologically normal features. These results in connection with the sharply increased antibody responses at 4th to 5th weeks p.i. of CY (Fig. 3) suggest that the cytotoxic activity of CY to lymphoid tissues was deleted at about 4th week p.i. of CY, when is approximately one week later than the guinea-pig system.[17] Pennington and Ehrie[20] have reported that an obvious histologic difference in lung tissues was seen between normal and CY-induced immunosuppressed animals after *P.aeruginosa* challenge. However,in the present study, it is hard to evaluate such cellular differences in the lungs infected with *P. multocida*,as no sequential quatitative histology was carried out.

Considering the high infectivity and recovery rate of *P. multocida* (PMC-1) in the CY-treated mice and rabbits, suppression of host immunity induced by CY treatment was evidently related to enhanced susceptibility of the animals with intranasal infection of *P. multocida*. Although more extensive study should be followed to elucidate humoral and cellular immune mechanisms associated with pathogenesis of intra-pulmonary *P. multocida* infection of the CY-treated mice and rabbits, the present data would support that host immunosuppression is closely correlated with the incidence and deteriorating symptoms of enzootic pasteurellosis of the rabbits.[11,19]

Conclusion

The influence of cyclophosphamide (CY) on susceptibility to experimental infection in the respiratory tracts of mice and rabbits with a strain of *Pasteurella multocida* isolated from a rabbit affected with enzootic pasteurellosis was studied. The mice and rabbits treated with doses of 300mg CY/kg and 150mg CY/Kg, respectively, showed enhanced susceptibility to intransal infection of *P. multocida*. The further assessed data indicated that CY treatment induced decreasing total numbers of leucocytes and mononucleated cells in peripheral blood, transient depletion of lymphocytes in spleens and depressing antibody responsiveness to the infected organism. Relationships between immunosuppression of the hosts and incidence of the respiratory diseases are discussed.

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Cyclophosphamide가 마우스 및 家兎의 *Pasteurella multocida* 人工感染에 미치는 影響

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抄 錄

Cyclophosphamide(CY)가 마우스 및 家兎의 *P. multocida* 野外分離菌株의 呼吸器管內 人工感染에 대한 感受性에 미치는 影響을 硏究하였다.

體重 Kg當 300mg CY를 注入한 마우스와 150mg CY를 處理한 家兎는 *P. multocida*의 呼吸器管內 人工感染에 대해서 높은 感受性을 보였다. 또한 CY 處理는 一時的으로 末梢血液 중에 있는 總白血球數와 單核白血球數의 減少를 惹起했고, 脾臟의 白髓와 赤髓에 있는 淋巴球의 消失을 招來했다. CY 注射된 家兎는 接種된 菌株에 대한 抗體形成能力의 低下를 나타냈다. 얻어진 結果는 家兎에 있어서 免疫機能의 低下와 呼吸器疾病 發生 間의 相互關係에 대해 考察하였다.