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Provocation of *Pneumocystis carinii* Pneumonia in Rats by Various Regimens and Routes of Steroid Administration

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Introduction

Pneumocystis carinii (*P.c.*) was noticed in human lungs first by van der Meer and Brug in 1942, though Chagas already found the organism in guinea pig lungs in 1909. Since then, *P.c.* pneumonia has drawn an increasing amount of attention in both clinical and medicozoological fields. Furthermore, it has become quite convenient to study *P.c.* pneumonia when Weller succeeded in inducing it in rats by giving cortisone acetate subcutaneously (1955, 1956). Disturbed immunosurveillance system in hosts caused by steroid therapy may evoke *P.c.* pneumonia. Later, Hendley and Weller (1971) successfully applied their concentration method to collecting *P.c.* cysts from animal lungs. The method might enable semiquantitation of the pneumonia. We tried to compare various schedules of steroid administration using various methods of detecting the organism. The provocation procedure originated by Weller and adopted by many other investigators is, however, very tedious and time-consuming. Water-insoluble cortisone acetate dissolved in various solvents is to be given subcutaneously at regular intervals. This procedure requires usually 5 to 8 weeks in order to induce maximum propagation of *P.c.* in rat lungs. In the present study, we attempted to induce *P.c.* pneumonia in rats by oral administration of steroid-incorporated diet as well as by other procedures thus far used, to harvest *P.c.* cysts efficiently from these lungs, and to quantitate pathological changes by the number of *P.c.* cysts detected.

Materials and methods

1) Rats: Wister rats were obtained from local breeders and were kept under conventional conditions during the study. Randomly 4 rats were kept in a metal cage of size 39×25×19 cm.

2) Steroid and antibiotics: Cortisone acetate (Wako Pure Chemical Industry, Tokyo, Japan), hydrocortisone sodium succinate (Solu-Cortef^{Rx}, Upjohn (Japan), Tokyo), or crude cortisol (Hoechst (Japan), Tokyo), was administered subcutaneously, intraperitoneally, or orally with drinking water or diet. Cortisone acetate suspension, 10%, was made according to Frenkel and Havenhill (1963). Tetracycline hydrochloride (Acromycin^{Rx}, Lederle (Japan), Tokyo) was given with drinking water *ad libitum*. Its concentration was routinely 0.5 mg/ml for initial 2 weeks and then 1.0 mg/ml until rats died or were sacrificed.

3) Routes of steroid administration

Subcutaneous route: 2 to 60 mg of cortisone acetate in suspension divided into 1 to 3 doses per week was injected subcutaneously to each rat regardless of its weight.

Intraperitoneal route: 60 mg of cortisone

acetate in suspension divided into 3 doses per week was given intraperitoneally to each rat.

Oral route with drinking water: Hydrocortisone sodium succinate was dissolved in water to make a concentration of 0.5 mg/ml. Tetracycline was also dissolved in the above solution to the concentration as previously stated. As drinking water, 160 ml of the resulted solution was supplied to 4 rats in a cage every second day. No other water was given. In this condition, the drinking water ran out completely by the time of the following supply.

Oral route with diet: The special diet was prepared by Oriental Kobo Kogyo Co., Tokyo, Japan containing crude cortisol in concentrations of 0.5 or 0.1 % and tetracycline in that of 0.2 %. Regular rat diet and the drugs were mixed in the appropriate concentrations. The mixtures were dried for 4 hours at 60 C which were cut into usual size of rat chow containing 6 to 8 % of water. The diet as well as tap water was provided *ad libitum*. No other tetracycline was given.

4) Methods for detection of *P.c.* cysts

All materials were obtained immediately after sacrifice of rats or within 24 hours of death.

Impression smears of the lung: Cross section of the diaphragmatic lobe of the right lung was wiped once gently with gauze, with which impression smears were made. The first imprint was served for enumeration of *P.c.* cysts.

Concentration procedure: The methods of Hendley and Weller (1971), and Ikai *et al.* (1977) was used with slight modifications. Briefly 1 g aliquot of the lung was minced with scissors, to which 20 ml of normal saline was added. The mixture was homogenized by a homogenizer (Nissei AM-5, Nihon Seiki Kaisha LTD, Tokyo, Japan) at 15,000 rpm for 5 min, which was filtrated through a metal filter of pore size of 76 μ. The filtrate was washed twice with normal saline by centrifugation at 1,000×G for 15 min. The resulted sediment was agitated with 2 ml of 0.1 % collagenase in normal saline at 37 C for 4 hours. Then 3 ml of normal saline was added to the suspension, 5 μl of which was transferred evenly to an area of 1 cm in diameter on a glass slide. After an appropriate staining, the number of *P.c.* cysts was determined under 400 magnifications. Cysts in 10 visual fields were counted when there were plenty seen, and those in 100 when there were few. The number of cysts equivalent to that in 1 g of the lung was estimated when the total weight of both lungs was less than 1 g.

Airway washing: A plastic catheter of 1 mm in diameter was inserted, as a rule, into the right main bronchus. Approximately 0.25 ml of normal saline was injected through the catheter into the lung and then aspiration was done once gently. Usually a minute amount of fluid was aspirated into the catheter, which was irrigated with 0.1 ml of normal saline. Then 0.01 ml of the resulted fluid was transferred to an area of 1 cm in diameter on a glass slide.

Tissue section: Tissue sections of the lung were served for detection of *P.c.* and staging of pathological changes according to Hughes *et. al.* (1973).

5) Staining: Grocott's staining (Luna, 1968) was adopted for enumeration of *P.c.* cysts in both tissue sections and smears, and Giemsa as well as Grocott's stainings for identification of *P.c.* Additionally, toluidin blue Q (Chalvardjian and Grawe, 1963), Gomori's, and/or PAS (Periodic-Acid-Schiff) stainings were applied to tissue sections, and toluidin blue O to impression smears and smears obtained by the concentration method and airway washing, if necessary.

6) Classification of histopathological changes of the lung: It was found convenient to categorize histopathological changes of rats into 3 stages according to Hughes *et al.*, although the classification was originally applied to *P.c.* pneumonia in children with malignancies.

7) Statistical analyses: Statistical analyses were performed by t-test, chi-square test, F-test and linear correlation and regression analysis.

Results

Table 1 shows effects of various schedules of subcutaneous cortisone acetate treatment on survivals of the rats studied. They were all 2.5 months of age at the start of the experiment. In the preliminary experiments, rats of age 1, 2.5 and 4 months were injected subcutaneously with 20 mg of cortisone acetate per 100 g of body weight 3 times weekly for 7 weeks. It was found suitable to use 2.5 months old rats in view of survival rates, economy, and bigger weight gap resulted between rats on steroid and on no steroid. Eleven groups were studied, each of which consisted of 4 or 8 rats. They were on either one regimen of 1, 10, 25, or 30 mg of subcutaneous cortisone acetate 1 to 3 times per week. Rats were tried to be kept on such treatments for 7 weeks, since 5 to 8 weeks of steroid treatment had been generally considered to be an optimal term for inducing maximum severity of *P.c.* pneumonia. All the rats of the groups on 30 mg of cortisone twice weekly and on 20 mg 3 times weekly died within 7 weeks. Three out of 4 rats on 20 or 25 mg twice weekly survived 7 weeks. The rats of other groups survived more than 7 weeks.

In Table 2, four methods—concentration method, impression smear, airway washing, and tissue section—were compared for detecting *P.c.* cysts from the lung. In this experiment, all rats except controls received subcutaneously 20 to 60 mg of cortisone acetate per week in 1 to 3 divided doses. Control rats received no steroid but tetracycline. Enumeration of cysts was performed with specimens obtained by the concentration, and with impression smears. Cysts were detected from all of the rats receiving more than 25 mg as a weekly dose by all of the methods. It is of interest that 12 out of 14 control rats (86%) were found to be positive for *P.c.* cysts in the lung applying the concentration. Number of cysts recovered from 1 g of lung tissue was $3.7\times10^5\pm4.1\times10^5$ (mean±SD). By the airway washing, 3 out of 14 controls (21%) were found positive.

Fig. 1 shows the relationship between subcutaneous cortisone doses and numbers of cysts detected from 1 g of lung tissue using the concentration method. These two factors were found to be correlated each other when cyst counts were transformed into logarithms ($r=0.571$, $P<0.01$). In addition, it was found preferable to give 30 to 50 mg of cortisone subcutaneously per week to a rat for inducing *P.c.* pneumonia. All of the rats receiving total of 60 mg weekly died within 7 weeks. There were no statistically significant differences noted in cyst counts between the control group and the group on 20 mg of cortisone per week.

Table 1 Survived days of rats on various schedules of subcutaneous cortisone injection

Cortisone dosage (mg/injection)	3 injections/week		2 injections/week		1 injection/week	
30	ND		28, 30, 41, 45days		*	(4/4)
25	ND		28days, Others sacrificed on 49th day.	(3/4)	*	(4/4)
20	21, 26, 28, 29, 29, 40, 44, 49days,	(0/8)	36days, Others sacrificed on 49th day.	(3/4)	*	(4/4)
10	*	(4/4)	*	(4/4)	ND	
1	*	(4/4)	*	(4/4)	ND	

All rars used were 2.5 months of age.
* All sacrificed on 49th day.
Numbers in parentheses denote those of rats living on 49th day of the treatment per total number of rats in each schedule.
ND: not done

Table 2 Four methods for detection of *P. c.* cysts from rats treated with subcutaneous cortisone acetate

Weekly dose of cortisone (mg/rat)	No. of rats studied	No. of rats positive for *P. c.* cysts in the lung by			
		concentration	impression smear	airway washing	tissue section
60mg	8	8	8	8	8
50	4	4	4	4	4
40	4	4	4	4	4
30	4	4	4	4	4
25	3	3	3	3	3
20	8	8(100)	7(88)	3(38)	5(63)
0	14	12(86)[1)]	8(57)[2)]	3(21)[3)]	5(36)[4)]

Numbers in parentheses denote percentages of rats positive for *P. c.* cyts in the lung in each group. Chi-square analyses

1) vs. 3) and 1) vs. 4) : $P<0.01$

2) vs. 4) and 3) vs. 4) : $P<0.05$

1) vs. 2) and 2) vs. 3) : nonsignificant

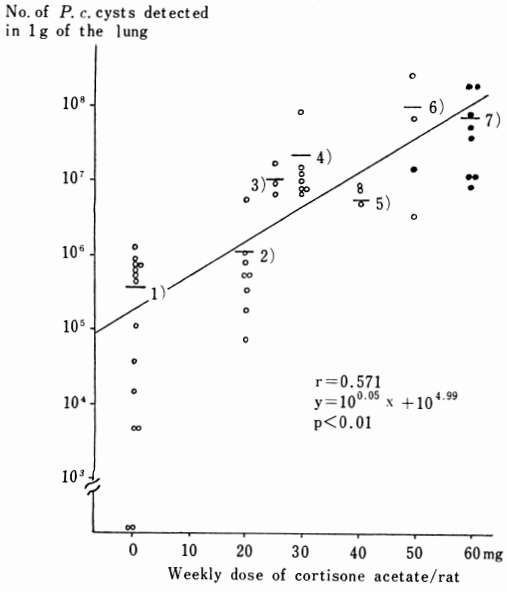

Fig. 1 The relationship between subcutaneous cortisone dosage and number of *P.c.* cysts detected by the concentration method.

Each solid circle indicates a rat died before 49th day of cortisone treatment.

Each bar indicates the mean of the group.

F-test

1) vs. 2), 4) vs. 6) and 4) vs. 7) : nonsignificant 2) vs. 4) and 2) vs. 7) : $P<0.01$

In the preliminary experiments, no statistically significant differences were found in numbers of cysts detected by the concentration method between male and female rats.

Table 3 summarizes the experiment in which rats were fed as much as needed on the experimental diet with crude cortisol and tetracycline. Three groups of rats were studied. The first group was on the diet containing crude cortisol in the concentration of 0.5% and the third in that of 0.1%. The second group was on the food consisted of equal amounts of 0.5% crude cortisol-incorporated and unincorporated diet (to be referred to 0.25% cortisol-incorporated diet hereafter). All rats on 0.5% and 0.25% cortisol-incorporated diets died within 21 days and 42 days respectively. The mean counts of cysts collected from 1g of lung tissue by the concentration method were 1.2×10^7 and 3.4×10^7 for these two groups respectively. The third group was divided into 5 subgroups, each of which consisted of 3 rats. Each subgroup was sacrificed at 5 weekly intervals. The experiment was started with 19 rats, 4 of which died between 17th and 21st day (75% survival rate). Those rats were excluded. Cyst count increased from mean of 1.2×10^7 in 1 week to

Table 3 Induction of *P. c.* pneumonia in rats fed on the diet containing crude cortisol

Cortisol content in 1g of diet	Rat used	No. of *P. c.* cysts in 1g of lung[1] ($\times 10^7$)	Term of steroid treatment (days) until died (d) or sacrificed (s)
5 mg	S-1	ND	12(d)
	S-2	ND	15(d)
	S-3	0.7	16(d)
	S-4	1.3	19(d)
	S-5	1.5	21(d)
	mean	1.2	
2.5 mg[2]	S-6	ND	16(d)
	S-7	2.6	32(d)
	S-8	1.5	39(d)
	S-9	6.1	42(d)
	mean	3.4	
1 mg	S-12	0.7	7(s)
	S-13	1.3	7(s)
	S-14	1.5	7(s)
	mean	1.2	
	S-15	1.1	14(s)
	S-16	1.2	14(s)
	S-17	2.7	14(s)
	mean	1.7	
	S-18	2.0	21(s)
	S-19	3.4	21(s)
	S-20	1.2	21(s)
	mean	1.9	
	S-21	4.7	28(s)
	S-22	3.8	28(s)
	S-23	5.9	28(s)
	mean	4.8	
	S-24	27.2	35(s)
	S-25	25.7	35(s)
	S-26	1.0	35(s)
	mean	18.0	

1) Concentration method applied.
2) Equal amounts of 0.5% crude cortisol-incorporated and unincorporated diet given.
ND : not done

that of 18.0×10^7 in 5 weeks.

Table 4 shows the effects of steroid treatments by 4 routes in view of cyst counts and histopathological changes. They were subcutaneous and intraperitoneal routes, and oral ones with drinking water and diet. Each group consisted of 4 to 6 rats. The first group was injected subcutaneously with 20 mg of cortisone acetate per rat 3 times weekly. The second group was injected in-

Table 4 Effect of various routes of steroid administration on provocation of *P. c.* pneumonia in rats

Route and Dose	Rats used	No. of *P. c.* cysts detected: Concentration method (in 1g of the lung)	No. of *P. c.* cysts detected: Impression smear[3]	Histopathology (stage)	Term of steroid treatment (days) until died (d) or sacrificed (s)
Subcutaneous cortisone 60 mg/rat/week	I-2	19.0×10^7	1,221	II	40(d)
	I-3	5.0×10^7	40	III	29(d)
	I-7	19.0×10^7	9,482	III	49(d)
	I-8	9.5×10^7	18,235	III	49(d)
	L-4	3.8×10^7	1,781	III	28(d)
	mean±SD	$11.1\times10^7\pm7.2\times10^7$ A)	6,152±7,380 C)		
Intraperitoneal cortisone 60 mg/rat/week	H-1	ND	35	I	23(s)
	H-2	ND	ND	I	35(s)
	H-3	ND	340	II	42(s)
	H-4	ND	136	I	49(s)
	mean±SD		170±251 D)		
Hydrocortisone in drinking water[1]	G-1	ND	39	II	35(s)
	G-2	ND	646	II	49(s)
	G-3	2.1×10^7	136	III	49(s)
	G-4	0.1×10^6	75	II	49(s)
	mean±SD		224±333 E)		
Crude cortisol in diet[2]	S-21	4.7×10^7	4,327	III	28(s)
	S-22	3.8×10^7	1,316	III	28(s)
	S-23	5.9×10^7	3,912	III	28(s)
	S-24	27.2×10^7	15,191	III	35(s)
	S-25	25.7×10^7	10,535	III	35(s)
	S-26	1.0×10^7	225	II	35(s)
	mean±SD	$11.4\times10^7\pm11.8\times10^7$ B)	5,918±5,787 F)		

1) 160ml (0.5mg/ml steroid concentration) supplied as drinking water every second day to 4 rats in a cage.

2) Diet containing crude cortisol in the concentration of 0.1% given *ad libitum*.

3) No. of *P. c.* cysts in 100 visual fields under 400 magnifications.

F test

A) vs. B) and C) vs. F) : nonsignificant

C) vs. E) and E) vs. F) : $P<0.01$

Linear correlation

A) and C) : $r=0.470$, $P<0.05$

B) and F) : $r=0.955$, $P<0.01$

traperitoneally with 20 mg of cortisone acetate per rat 3 times weekly. The third group was given Solu-Cortef^{Rx} with drinking water in the concentration of 0.5 mg/ml. Tetracycline was also mixed with the water in the concentrations as stated earlier. Every second day, 160 ml of this fluid was supplied to 4 rats in a cage. The fourth group was given 0.1% cortisol- and 0.2% tetracycline-incorporated diet *ad libitum*. A larger number of cysts were detected in impression smears of lungs obtained from rats treated with subcutaneous steroid injections or steroid-incorporated diet than from those treated with intraperitoneal steroid injections or steroid containing drinking water. There were no statistically significant differences noted between the first two treatments in terms of cyst counts of both concentrated materials and impression smears. With the steroid-incorporated diet, more cysts seemed to be induced for a shorter period of time compared to other routes of steroid administration. Furthermore, more advanced histopathological changes were observed with subcutaneous steroid treatment or steroid containing diet. It was found more than anything else that treatment with steroid-incorporated diet was far more convenient without troublesome processes, time-saving and rather economical for provocation of *P.c.* in rat lungs than with any other methods currently used.

Discussion

Experimental *P.c.* pneumonia in rats is induced usually according to Frenkel *et al.* (1966), and Barton and Campbel (1969). Total weekly dose of steroid given and the term of steroid treatment seemed to be the two important factors in order to provoke maximum degree of the pneumonia or to obtain maximum propagation of *P.c.* These investigators gave 25 mg of cortisone acetate subcutaneously twice weekly for 5 to 8 weeks, which has been most widely adopted by others. The term of such steroid treatment was considered to be the most appropriate period of time to induce the pneumonia in rats. Various observations have been used as indices to estimate the progress or severity of the experimental pneumonia including weight changes of rats, and their survivals as well as pathological changes of the lung, and numbers of *P.c.* cysts detected in impression smears. Hendley and Weller (1971), and Ikai *et al.* (1977) have introduced concentration methods to collect *P.c.* cysts.

In our study, treatment with 30 to 50 mg of subcutaneous cortisone acetate per rat per week was found to be suitable to provoke *P.c.* in the lung. The doses are comparable to that adopted by previous workers.

We, then, examined four methods for detecting *P.c.* cysts which included concentration procedure and airway washing as well as impression smear and tissue section. All of four methods were able to detect *P.c.* cysts from lungs of all rats injected subcutaneously with more than 25 mg of cortisone acetate per rat per week. Although the method of airway washing in our study is quite different from the bronchial lavage procedure or tracheal washing used in the clinical area, it is the only currently available fairly non-invasive method to diagnose the pneumonia. We hope that the airway washing would serve as an experimental model of the tracheal washing. It will be of interest to examine the relationship between results obtained by this method and histopathological changes of both human and animal lungs. Clinically, sputum specimens would be most convenient materials for the diagnosis (Gajdusek, 1957; Fortuny *et al.*, 1970) though they have been considered not to be suitable ones because of low detection rates of *P.c.* from them. Thus, it may be of clinical importance to attempt to apply a concentration method to sputum specimens for increasing efficiency of detection and diagnosis of *P.c.* pneumonia. In addition, it is interesting that *P.c.* cysts were detected in 86% of control rats by the concentration method, and in 21% even by the airway washing, seemingly the least efficient method of the four. This might

suggest possibilities of overdiagnosis of the pneumonia. In this sense, quantitation of *P.c.* in the lung, if possible, could give a more accurate diagnosis both in man and in animals.

In order to provoke *P.c.* pneumonia more easily, we attempted to feed rats on cortisol-incorporated diet. Intraperitoneal (Linhartová, 1956) and intramuscular (Sheldon, 1959) administrations of steroid, and oral administration with drinking water (Hendley and Weller, 1971) have been tried by others. The last two of them were found to be as effective as subcutaneous treatment in inducing *P.c.* pneumonia. These methods, however, are all tedious, time-consuming and expensive. We are unaware of previous reports of oral administration of steroid-incorporated diet.

We used cortisone acetate subcutaneously and intraperitoneally, hydrocortisone sodium succinate orally, and crude cortisol orally in the present study. Detailed data are not available to us about potency and purity of the crude cortisol. Furthermore, injectable form of hydrocortisone (sodium succinate derivative) which was given orally is not readily compared with orally administered crude cortisol in view of potency or efficacy in propagation of *P.c.*

It was found that the oral administration of the crude cortisol was as effective as subcutaneous injection in inducing *P.c.* pneumonia. Rats were left to be fed on the special diet as much as needed without trouble. After 35 days of such feeding, more than 10^8 of *P.c.* cysts were detected from 1 g of lung tissue, which was comparable to the results obtained after an optimal period of subcutaneous treatment. So were the histopathological staging of the pneumonia. *P.c.* propagated themselves explosively from the mean of 37×10^4 to that of $1{,}200\times10^4$ in a week of the cortisol-incorporated diet treatment. Cyst count began to increase steeply at 4 weeks of such treatment reaching $1{,}800\times10^4$ in 5 weeks. This pattern of *P.c.* propagation could be explained by the extent of disturbance of host immune system by the treatment. More studies, however, are essential for any conclusions. The life cycle or mode of *P.c.* propagation is not fully understood currently. We believe the oral administration of steroid used in our study will serve as the best simple procedure thus far known to provoke *P.c.* pneumonia or propagate *P.c.* in rat lungs.

Abstract

Pneumocystis carinii pneumonia was induced in rats by various schedules of corticosteroid administration. Quantitative estimation of the pneumonia was attempted by counting cysts collected by concentration or those in imprsesion smears of the lung. Specimens obtained by airway washing of the lung, and tissue sections were also examined. Total weekly dose of 30 to 50 mg of subcutaneous cortisone acetate was found to be an optimal dose for inducing the pneumonia in terms of survivals of rats and cyst counts of *Pneumocystis carinii* in the lung. This treatment was continued for 7 weeks. Weekly dose of cortisone acetate showed a significant correlation with counts of cysts collected by concentration ($r=0.571$, $P<0.01$). Control rats on no steroid treatment were found to have $3.7\times10^5\pm4.1\times10^5$ of cysts in 1 g of lung tissue. Weekly dose of either one of 30, 40, 50 and 60 mg was found to provoke or propagate a significantly larger number of cysts compared to no steroid treatment ($P<0.01$). Examination of the concentration method, impression smear, airway washing and tissue section for detecting *Pneumocystis carinii* cysts from the same specimen revealed that they were detected by either one of four methods in all of rats thus treated.

In addition to subcutaneous steroid injection, compared were intraperitoneal cortisone acetate injection, and oral administration of hydrocortisone sodium succinate in drinking water and crude cortisol in diet. Oral administration of crude cortisol mixed with diet in a concentration of 0.1% was found to be as effective as subcutaneous

cortisone treatment in provoking the pneumonia. This required a shorter period of time for *Pneumocystis carinii* to propagate in rat lungs. It was found that oral administration of steroid-incorporated diet served as an quite effective, time-saving and economical method to induce the pneumonia in rats.

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ラットにおける種々のステロイド療法による *Pneumocystis carinii* 肺炎の誘発

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我々はステロイドによる実験的 *Pneumocystis carinii* (*P.c.*) 肺炎の誘発を種々の投与法及び投与量にて行ない，各種の検出法を応用して比較検討した．

ステロイド剤の投与は酢酸コーチゾンの皮下及び腹腔内投与，ハイドロコーチゾン・ソディウム・サクシネートの飲料水混入投与，粗コーチゾールの固型飼料混入投与によつた．

検出については，肺の集シスト法により得られた材料，気管支洗浄液，肺のスタンプ標本，及び肺組織切片標本を用い，これらからの *P.c.* シスト検出を同時に試みた．

酢酸コーチゾンの皮下注射により誘発された *P.c.* を集シスト法により検出した結果，投与量とシスト数の間に強い正の相関を認めた ($r=0.571$, $P<0.01$)．更に，一週間当りのコーチゾン投与量がラット一匹当り 30 mg を越えると検出されるシスト数に関して非投与群との間に有意な差を認めたが ($P<0.01$)，60 mg を越えると投与開始7週間以内にすべて死亡した．一般にラットに於ては5～8週がステロイドによる *P.c.* 肺炎誘発の至適期間と考えられている．このため皮下投与により効率よく *P.c.* 肺炎を発誘させるのには30～50 mg が至適量と考えられる．一方非投与ラットの肺にも集シスト法の応用により1g当り平均 3.7×10^5 の *Pc.* シストを認めた．

P.c. シスト検出に関しては集シスト法の応用及び肺のスタンプ標本からの検出率が他の方法より統計的に効率の良いことが認められたが，一週間当り 25 mg 以上の酢酸コーチゾン皮下投与ではこれらすべての方法で100％の検出率を得た．

種々投与法の比較検討の結果，粗コーチゾール0.1％，テトラサイクリン0.2％混入の固型飼料投与により *P.c.* 誘発が効率よく起ることが判明した．すなわち，投与開始一週間で *P.c.* シスト数が肺1g中，平均 1.2×10^7，5週に至つては平均 18.0×10^7 であつた．従来行なわれている至適条件下での皮下注射による誘発法と検出されたシスト数に関しては統計的に有意な差を認めず，加うるに労力がはるかに少く，早期に多数の *P.c.* を誘発でき，かつ経済的であつた．これよりコーチゾールの固型飼料混入投与は今後の実験的 *P.c.* 肺炎誘発に極めて有効と考えられる．