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Studies on the prevention of tuberculosis in pet dogs 1. The effects of BCG pretreatment in pet dogs inoculated experimentally with *Mycobacterium bovis*

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애완견의 결핵예방에 관한 연구 1. *Mycobacterium bovis*를 실험적으로 접종한 애완견에 있어서 BCG의 전처치 효과

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초록 : *Mycobacterium (M) bovis*를 인공감염시킨 개에 있어서 BCG의 전처치효과를 *in vivo* 및 *in vitro*에서 검토하였다. 개들은 BCG 전처치군, *M bovis* 단독처치군, 비감염대조군의 세군으로 나누었다. BCG는 *M bovis* 복강접종 3주일전에 0.2㎖를 피내접종하였다. 결핵균 투여 4개월후에 전군을 도살하여 실험에 사용하였다. 도살시 모든 처치군에서 감염이 확인되었다. 병리조직학적으로 BCG전처치군의 폐장내에서는 경도의 macrophage의 침윤과 소상의 육아종 형성이 관찰되었으나 *M bovis* 단독처치군에 있어서는 보다 고도의 macrophage의 침윤, 중등도의 호중구의 침윤 및 중등도의 육아종의 형성이 확인되었다. 각 동물의 기관지폐포세정액을 분리하여 그 속의 총세포수와 각 세포의 분획을 검토하였다. 비감염 대조군의 기관지폐포세정액내의 총세포수는 두 처치군보다 훨씬 낮았으며 *M bovis* 단독처치군의 총세포수는 BCG 전처치군보다 1.8배 높았다. 이 세정액으로부터 폐포 macrophage를 분리배양하여 macrophage의 활성능과 결핵균의 증식능을 관찰하였다. BCG처치군은 *M bovis* 단독처치군에 비하여 높은 Fc receptor 활성(rosette 형성능, 탐식능)과 낮은 결핵균의 증식이 관찰되었다. 그러나 BCG의 전처치는 결핵균을 killing하지는 못하였다. 개에게 BCG를 전처치하면 폐내에 극소수의 결핵균이 지속적으로 잔존하지만 폐포 macrophage는 이미 항결핵성면역능을 지닌채로 계속 활성화된 상태로 존재하기 때문에 결핵에 대하여 예방효과를 갖는다고 사료된다.

Key words : BCG, *M bovis*, alveolar macrophages, Fc receptor activity, protective effect.

Introduction

Tuberculosis is an infectious disease caused by mainly *Mycobacterium(M) tuberculosis* and which has been an important world-wide public health problem. It estimated that there are approximately 726 thousand cases of tuberculosis in Korea and 95 thousand of theirs were infectious patients.[11] In recent year, mycobacterial disease is of renewed interest because of the increase in *M tuberculosis* disease, in part due to acquired immune defiency syndrome(AIDS)[6,16,18] and because of the prominence of *M avium complex* infections in AIDS patients.[1] First of all, prevention against infectious

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route is most important for the eradication of tuberculosis. Among the host of tuberculosis, the dog has known to more susceptible to *M bovis* and *M tuberculosis*. Furthermore, according to the increases of breeding of pet dogs in apartments, the opportunity to contact to tuberculosis persons goes on increasing every year. However, tuberculosis infected dogs does not show any symptoms, the dog again transmiss tuberculosis to man. Therefore, perevention of tuberculosis in dogs will be important for the elimination of infectious route in human. However, the survey about tuberculosis infection in dogs is still unknown. For prevention of tuberculosis, Bacilli Calmette-Gue'rin(BCG) vaccine has been used in recent years. However, the preventive effect of BCG were different from 2 - to 90% according to difference of vaccined strain, vaccine dose, and infectious frequency of atypical mycobacterial disease.[13] The mechanism of protection in tuerculosis is believed to nonspecifically stimulate the host' resistance against various target.[8] But little attention has been focused on the possible role of BCG effect elevating mononuclear phagocytes in dogs. Thus, more reliable means of assessing the BCG reactive status of dogs would be of considerable value.

Bronchoalveolar lavage(BAL) has been used to evaluate the kinetics of progressive pathologic events and immunologic phenomena.[5] Among the BAL, the pulmonary alveolar macrophages(AM) constitute the first line of defense against an aerogenic mycobacterial challenge.[3] *M bovis* and *M tuberculosis* are intracellular pathogens capable of persisting and replicating within nonactivated macrophages.[4,10] For this reason, the subpopulation of BAL, the intracellular multiplication of organisms and the activity of alveolar macrophages were examined. In an attempt to develop new diagnotic and vaccine tools for the control of canine tuberculosis, we study that the effect of BCG pretreament in dogs infected with *M bovis* were explored *in vivo* and *in vitro*.

Materials and Methods

Animals : Six mongrel dogs of both sexes originated from same maternal dog, about 1 month of age and weiging 1000 to 1200 g were obtained from a commerical supplier. These animals were checked for being negative for tuberculosis detection by X-ray. They were given Purina Dog Chow-(Seoul, Korea) and water *ad libitum* until use.

Experimental design : Dogs were divided into 3 groups of 2 each ; BCG pretreatment group, *Mycobacterium(M) bovis* only treatment group, and uninfected control group. The BCG pretreated group were sensitized intradermaly with 0.2㎖ of BCG(Japan BCG Co.) before 3 week of *M bovis* treatment. The organisms(*M bovis*) were intraperitoneally injected as concentration of about 1×10^7 per dog. All dogs were killed 4 months after injection.

Bacteria : *M bovis* were obtained from the Jung-Ang infectious disease Insitute(Dae Jun, Korea). They were grown in modified Sauton medium with added Tween 80 as described previously and stored at 37℃ until required.[14]

Culture media and reagents : RPMI 1640 complete medium was prepared from powder mix(Nissui Seiyaku Co., Tokyo, Japan) and supplemented with fresh L-glutamine-(0.3㎎/㎖, Wako Chemical, Osaka, Japan), 2-β-mercaptoethanol(50mM, Wako Chemical), HEPES(15mM, Sigma Chemical, St Louis, Mo), sodium pyruvate(1mM, Wako Chemical) Fetal calf serum(FCS, Gibco, Grand Island, NY) was inactivated by heating at 56℃ for 30minutes and added to 10% concentration. Sheep red blood cell, SRBC(Nippon Bio-Supp. Center, Japan) were stored in Alserver's medium. The anti-SRBC IgG antibody(Cordis Laboratories, Miami, U.S.A.) was used.

Isolation of alveolar macrophages : Bronchoalveolar cells were obtained by bronchoalveolar lavage(BAL) as described.[12] Briefly, after the animals were killed, the jugular vein was severed and the trachea were cannulated. A total volume of 500㎖ of Ca^{+2}, Mg^{+2} free phosphate buffer saline PBS(-) was infusd in each dog, about 90%(450㎖) of which was recovered. The bronchoalveolar cells were obtained by centrifugation at 1500rpm at 4℃ for 10minutes, washed three times in PBS(-) and finally resuspended in RPMI 1640 medium(Nissui Co. Japan). Then cells were counted in a hemocytometer chamber and viability was determined by trypan blue exclusion. For comparision, values of total cell counts in lavage fluids was corrected for the corresponding fluid recovery and all values were calculated as cell counts per 450㎖ of lavage fluid recovered. The resuspended cells were adjusted to 1×10^6 viable cells in 1㎖ medium and 2㎖ of the suspension was placed onto 35㎖ plastic dishes(Corning ; Iwaki glass, Tokyo) with cover glass. After incubation at 37℃ for 2hrs, nonadherent cells were removed by washing three times, remaining adherent cells were further cultured with the RPMI 1640 complete medium without antibiotics. At various time intervals, the coverslips were washed three times with PBS(-), fixed with methyl alchol and were stained by Wright-Giemsa solution or Ziehl-Neelsen method for acid-fast bacilli.

Anaysis of bronchoalveolar cells : Differential counts of lavage cells were made from cytocentrifuge smears(Tomy Seico Co., LTD, Tokyo) prepared with 3×10^4 cells, air dried, fixed and stained with Wright-Giemsa solutions(Sigma). A differential count was performed using commonly accepted morphological criteria; percentage were determined by counting of 500 to 1000 cells.

Fc receptor mediated activity of alveolar macrophages : Fc receptor activity of alveolar macrophages was tested by a modified method of Wright and Silverstein.[17] The adherent monolayers of BAL on coverslips were prepared by incubation for 2hr at 37℃. Sheep red blood cells(SRBC) were washed three times with PBS(-), resuspended, and sensitized with a 1 : 256 dilution of rabbit anti-SRBC IgG antibody(EA) for 30min at 37℃ in shaking water bath. After washing three times, 100 $\mu\ell$ of 5%(V/V) sensitized SRBC was added to each adherent monolayers containing 1 $m\ell$ -RPMI complete medium. For rosette assay, the coverslips were incubated for 30min at 4℃ and dipped into PBS(-) to remove unbound EA, dried, washed, fixed and stained with Wright-Giemsa stain. Data are presented as attachment index, the number of EA bound per 200 alveolar macrohages. Fc-mediated phagocytosis(37℃, 1hr incubation) was scored in a similar fashion after uningested EA were lysed by brief exposure to 0.83% ammonium chloride.

Peripheral blood monocyte culture : Blood obtained from jugular vein was collected in heparinized syringes. Diluted blood(1 : 3 in RPMI medium) was layered on Ficoll-paque gradients(ρ=1.077 g /$m\ell$; Pharmacia Inc., Uppsala, Sweden). The interphase containg mononuclear cells was then washed four times with PBS(-) and purified by adherent for 2hrs. After 5days, the coverslips were washed three times with PBS(-), air-dried, fixed and stained by Wright Giemsa solution.

Histopathology : After lavage, the lungs were removed and each lobe was fixed by immersion in 10% neutral buffered formalin, embedded in paraffin and 4 μm-sections were made and stained by hematoxylin and eosin(H & E) and Zieh1-Neelsen method for acid-fast bacilli.

Statistical analysis : Results are expressed as mean values ±SEM. Statistical significance was determined using Student's unpaired t-test($p<0.05$).

Result

Histopathological finding : At four months after *M bovis* inoculation, all dogs were killed and examined. Gross changes were only limited to the lung. *M bovis* only treated dogs had the nodules of 1~3cm in the dorsal part of various lobes-(Fig 1). The cut surface was uniform. However, no significant alteration in the BCG-pretreament group noted(Fig 2). Histopathologically, pulmonary lesions from *M bovis* only treated dogs were characterized by diffuse marked infiltration of macrophages mainly and by a cluster of polymorphonuclear(PMN) cells occasionally(Fig 3). Typical granulomas were not as common. The nonspecific granulation tissues were composed principally of epitheloid cells surrounded by thin fibrous tissue in which were scattered small collections of macrophages and lymphocytes, but Langhan's giant cells were rare(Fig 4). In BCG pretreated dogs, the pulmonary lesions were similar to *M bovis* only infected dogs. However, in BCG pretreatment dogs, the magnitude of the tuberculous granulomas was more smaller and only a slight infiltration of alveolar macrophages but not PMN cells was observed-(Fig 5). In both groups, the presence of *M bovis* in the lung tissue was difficult to find precisely because the organisms were too short bead form and seen rarely(Fig 6).

Influx and fractionation of BAL(bronchoalveolar lavage) : The mean total nucleated cell count and differential cell count data from BALs were as shown Table 1. The number of lavage cells harvested from lungs of uninfected dog was lower than that of both treated groups. The *M bovis* only treated dogs showed the most singnificant increase in the number of total lavage cells(Table 1). The most fractions of BAL was alveolar macrophages(85%) in both the treated groups(Fig 7). The proportion of macrophages was determined by the phagocytosis of IgG-coated SRBC in BALs or by their morphological criteria. The number of neutrophils in the BCG pretreaatment group was significantly lower than that from *M bovis* only group. In contrast, the total number of alveolar macrophages from the BCG pretreament group was more than that of *M bovis* only treated group.

Fc receptor mediated activity : Fc rosette and Fc receptor mediated phagocytosis were evaluated by incubating alveolar macrophages with IgG-coated sheep RBC at 24hr culture. The results were summerized in Table 2. As shown in Fig 8, 9, the values of ingestion and attachment in the BCG pretreament group were sigificantly higher than that of the *M bovis* only treated group and control group.

Observations of cytopathic effect(CPE) and proliferation of *M. bovis* : The apperances of CPE in the alveolar macrophage monolayers isolated from BALs were checked at

Table 1. Bronchoalveolar cells analysis

Characteristics	*M bovis* only treated group	BCG pretreated group	Control group
Total cell yield, $\times 10^8$	2.01±0.4	1.1±0.2	0.7±0.3
Viability, %	97.3±0.4	97.5±0.3	98.0±0.4
Macrophages*	85.8±0.3	88.7±0.2	91.5±0.1
Lymphocytes*	5.6±0.3	5.8±0.4	4.1±0.3
Neutrophils*	7.2±0.2	5.3±0.2	4.3±0.2

*: Differential counts(% of total cells). Each value represents the mean of two cell preparations±SEM.

Table 2. Fc receptor mediated rosette and phagocytosis of IgG coated sheep RBC by alveolar macrophages(AMø) cultured from various dogs.

Source	Fc rosette			Fc phagocytosis		
	AMØ with bound SRBC(% of total)	Bounded SRBC (mean/AMØ)	Index	AMØ with ingested SRBC(% of total)	Ingested SRBC (mean/AMØ)	Index
M bovis only	34.8	12.2	424.5	59.5	6.7	398.6
BCG pretreated	47.4	12.9	611.0	72.9	10.1	729.2
Un-infected control	31.6	11.0	347.6	53.5	6.5	347.1

* : percentage of total AMø with bound or ingested red cell × mean number of red cells bound or ingested/AMø.

different intervals. The bacilli of long-beaded form were seen in cultures of both the treated groups. However, at 7days after cultures the replicaxion of *M bovis* in alveolar macrophages from BCG pretreatment group was markedly delayed as compared with that of *M bovis* only group(Fig 10).

Analysis of peripheral blood cells and culture of monocytes : In the culture of peripheral blood monocytes, the BCG pretreated group produced a marked bastic cell morphology as compared with that of *M bovis* only treated group(Fig 11, 12).

Discussion

A more recent 2 year survey of Korean National Tuberculosis Association reported that the prevalence of tuberculosis was 1.67% of total populations and it is a leading cause of death(killing more than 5,000 persons each year) in Korea.[9] Therefore, until recently, tuberculosis has known to a important epidermiological disease threating our health.

Among the host of tuberculosis, the majority of canine cases have known to be caused by the humen type. Accordingly, because mostly infection contracted from tuberculous owner and disease is again transmitted from dog to man, the diagnosis and prevention of tuberculosis in the dog could be important for the elimination of TB infectious route. However, little attention has been focus on animal model as dog having the most close friendly relation with human. More recently, Kim[7] reported the result that 6 cases of 70 dogs breeding in household were infected to tuberculosis. Tuberculin skin tests have been used in the diagnosis of tuberculosis in human and other animals. However, it is not a reliable tool for canine tuberculosis detection. As the diagnosis of tuberculosis in the dog, the inoculation of BCG have known to be reliable but little is known about the effect of BCG in the dog.

The initial step in the pathogenesis of bacilliary pulmonary tuberculosis(BPT) has known to the establishment of the primary lesion. Many disagreements are whether tubercle bacilli reach the apical-subapical(A-SA) region of lung via the airstream or the blood stream. In our observations, *M bovis* infection was readily established in all the intraperitoneally(i. p) inoculated dogs and the infection was confirmed by the individual necropsy finding and the presence of the organisms in the alveloar macrophage cultures. Therfore, this result suggest that the tubercle bacilli are able to transport from the peritoneal cavity to the A-SA region via the bloodstream by i.p. injection. The BCG injection produced focal granuloma fromation and mild macrophage infiltration as compared with those of the *M bovis* only treated group. Thus, the alveolar macrophage migration in the lung may be related to the magnitude of microorganism multiplication, However, because the slaughter of dogs were achieved at early time as 4 months after infection, the in-

fected dogs would be developed mild multifocal granulomatous pulmonary lesion. As this result, we think that the infected dogs would have progress toward a more severe form of tuberculosis if they were kept alive longer and administered by the intratracheal route.

Histopathologically, it is difficult to precisely find the presence of the organisms in the lung tissue. However, *In vitro* we are able to readily find the presence of *M bovis*. This result is consist with the result the acid-fast organisms in lung tissue could be either destroyed or present in a cell wall-deficient state[2] but *in vitro*, alveolar macrophages may povide an excellent environment for replication of *M bobis*. In our study, because we used the peritoneal route, perhaps the peritoneal macrophages play a role in clearing the entry of *M bovis* into the lung of dog. However, *M bovis* show strong affinity for alveolar macrophages *in vitro* as compared with peritoneal macrophages(data not shown). It suggested that the organisms are taken up by alveolar macrophages rather than by peritoneal macrophages located at injection site. At 7 days after alveolar macrophage culture the *M bovis* only treated group has the rapid cell destruction and high intracellular multiplication of *M bovis*. These *in vitro* results suggest that *M bovis* is ingested by an alveolar macrophages at the site of implantation and after a lag period of a few days, the mycobacteria multiplies intracellularly. Then, the macrophage dies and the organisms released are ingested by other un-infected macrophages. However, the BCG pretreatment produced the inhibition of the intracellular multiplication of *M bovis* and the high Fc receptor mediated activity although they were incapable of the killing the organism. These results suggest that the mycobacteria within the lung may remain in a matabollically active state in order to induce the maxium mononuclear cell response of the type associated with acquired antituberculosis immunity. Accordingly, one possible reason for the protective effect of BCG may be related to the phagocytosis enhancement and the inhibition of intracellular replication of mycobacteria to some extent.

The protective mechanism of BCG against tuberculosis is believed to be one of cellular immunity.[8] Recently, it seem likely that BCG may trigger the monocyte activation to produce TNF-α through a phagocytic process. In this work, although the mechanisms mediating the growth inhibition of mycobacteria by activated macrophages has not been elucidated, the effective mechanism of BCG could be related to the opinion of Takashima et al.[15] suggesting that the levels of TNF-α produced by monocytes were related to the disease states of pulmonary tuberculosis.

In this study, we supposed that there is a correlation between the severity of experimental *M bovis* infection in dogs and the number of inoculated organisms. Moreover, the protective effect of BCG can be proportional to the size and the extent of the pulmonary lesions. However, based on clinical, histological, and cultural criteria, we found the practical dose of BCG in canine resistance against tuberculosis remains to be determined because of a complex pattern of involvement by a host's defense mechanism. Furthermore, the problem of this experiment may be due to variety of factors such as infectious dose of *M bovis*, dose of vaccined BCG, and injected route.

In conclusion, we suggest that the BCG may be effective for the reduction of the lung lesion and bacilli multiplication in the dog. Further studies will be need to support results that dogs vaccinated with BCG produce a resistance against tuberculosis.

Summary

Dogs were divided into 3 groups of two each; Bacillie Calmette-Gue'rin(BCG) pretreatment, *M bovis* only treatment and uninfected control group. BCG were vaccinated intradermally with 0.2㎖ before 3weeks of *M bovis* intraperitoneal infection. Infection at necropsy 4months later was readily in the both treated dogs. Histopathologically, the BCG pretreated dogs produce the moderate accumulation of macrophages and focal granuloma formation in the lung, whereas the *M bovis* only treared dogs produce the accumulation of predominantly macrophages, occasionaly polymorphonuclear cells and the more larger granuloma. Bronchoalveolar lavage(BAL) was obtained and total and differential cell counts were examined. Total number of BAL cells harvested from uninfected dogs is lower compared with those of the both treated groups. The total cell number of *M bovis* only treated dogs were singificantly higher 1.8 times than that of the BCG pretreated dogs. The Fc receptor activity and the growth of organism in alveolar macrophages obtained from BCG pretreated dogs were compared with that in macrophages from *M bovis* only treated dogs. BCG vaccination resulted in substantial macrophage activation, measured as increased Fc receptor mediated phagocytosis and rosette formation, as wells as the inhibition of intracellular mycobacteria multiplication. However, actibated macrophages taken from BCG pretreated dogs are incapable of

killing the *M bovis*. Thus, these results suggest that BCG pretreatment in the dog may produce a protective effect against tuberculosis because active alveolar macrophages have acquired antituberculous immunity, although few mycobacteria within the lung remain in a metabolically active state.

Legends for figures

Fig. 1. Gross appearance of lung from *Mycobacterium bovis* only treated dog.
The dog had the nodules of 1~3cm in the dorsal part of various lobes.

Fig 2. Gross appearance of lung from BCG pretreated dog.
The marked lesions were not observed.

Fig 3. Microscopic apperance of lung from *M bovis* only treated dog.
The lesion were characterized by diffuse marked infiltration of alveolar macrophages mainly and by a cluster on neutrophils occasionally. H & E. × 200.

Fig 4. Microscopic appearance of lung from *M bovis* only treated dog.
The nonspecific granulation tissue was composed principally of epitheloid cells, the small collections of macrophages and lymphocytes, but giant cells were rare. H & E. × 400.

Fig 5. Microscopic appearance of lung from BCG pretreated dog.
The morderate infiltration of alveolar macrophages was observed. H & E. × 200.

Fig 6. Microscopic appearance of lung form BCG pretreated dog.
The organisms of short and bead from were observed in the alveolar macrophages. Ziel-Neelsen. × 400.

Fig 7. Cytocentrifuge preparation of Bronchoalveolar lavage(BAL) cells from *M bovis* only treated dogs. Macrophages were approximately 85% of total BAL cells. Wright-Giemsa. × 400.

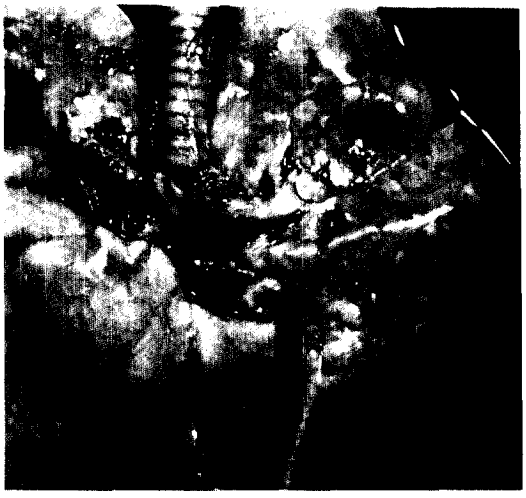
Fig 8. Alveolar macrophages cultured from *M bovis* only treated dogs.
The low rosette formation was observed as compared with that of the BCG pretreated dogs. Wright-Giemsa. × 200.

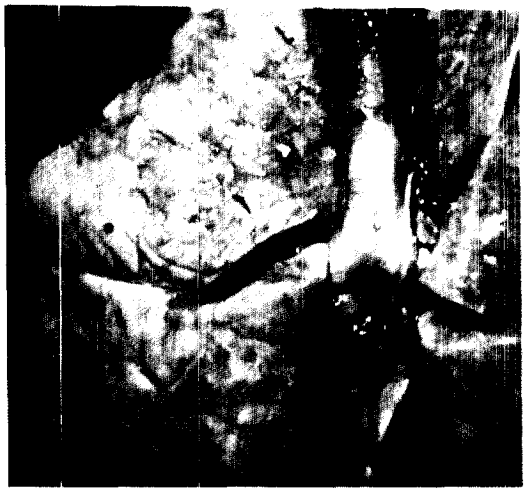
Fig 9. Alveolar macrophages cultured from BCG pretreated dogs.
Note exaggerated rosette formation of immunoglobin-coated sheep erythrocytes. Wright-Giemsa. × 200.

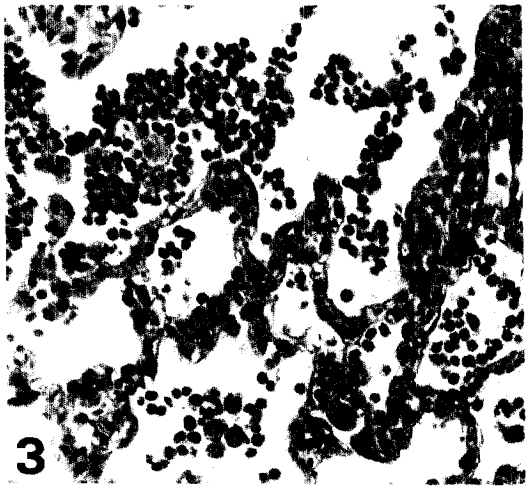
Fig 10. Long, slender rod-shaped organisms in alveolar macrophages cultured from *M bovis* only treated dogs. Ziel-Neelsen. × 1000.

Fig 11. Morphology of 2day cultured monocytes from the peripheral blood of *M bovis* only treated dogs. More smaller monocytes were observed as compared with that of BCG pretreated dogs. Wright-Giemsa. × 400.

Fig 12. Morphology of 2day cultured monocytes from the peripheral blood of BCG pretreated dogs. The monocytes of marked blastic type were observed. Wright-Giemsa. × 400.







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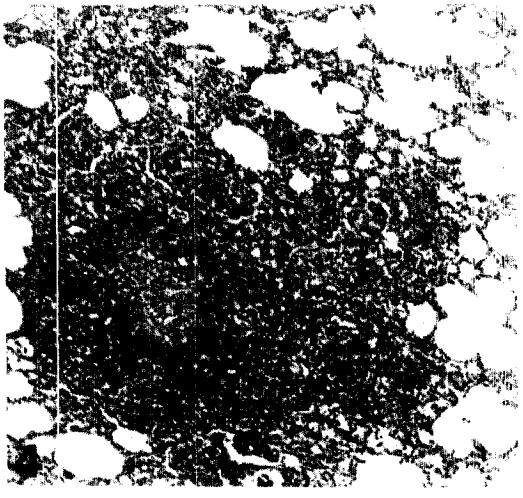

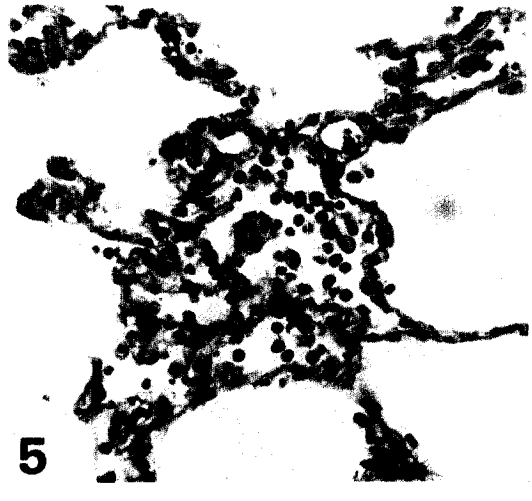

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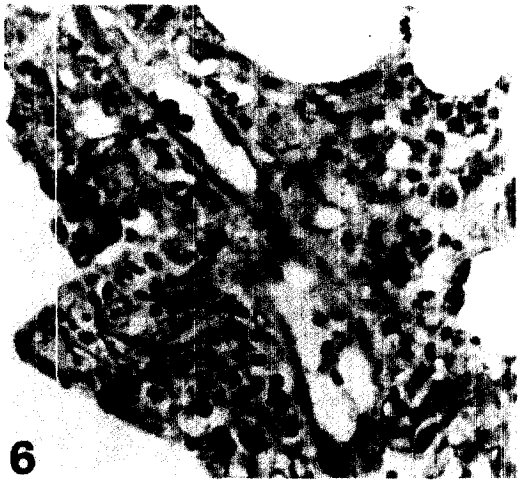

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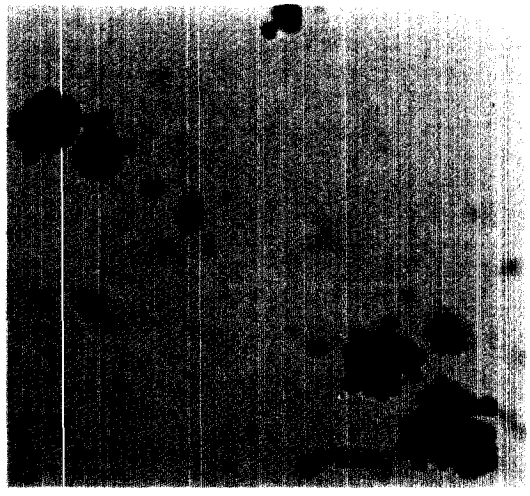

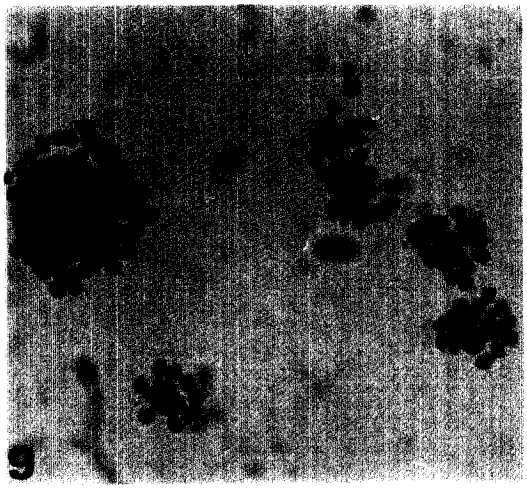

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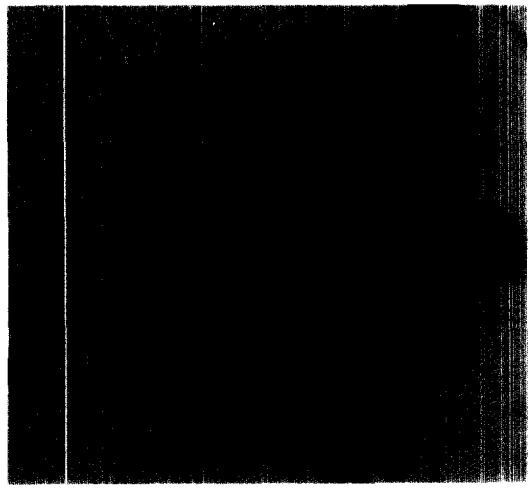

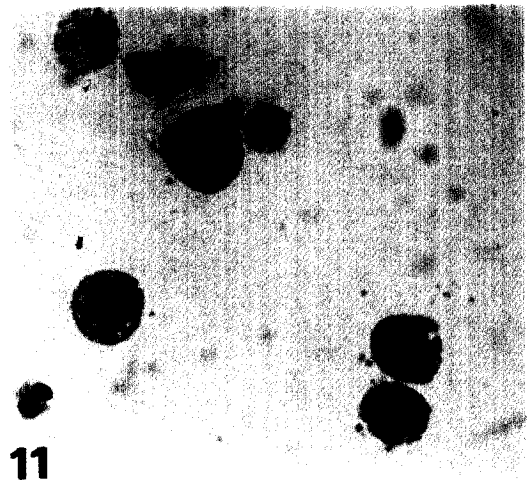

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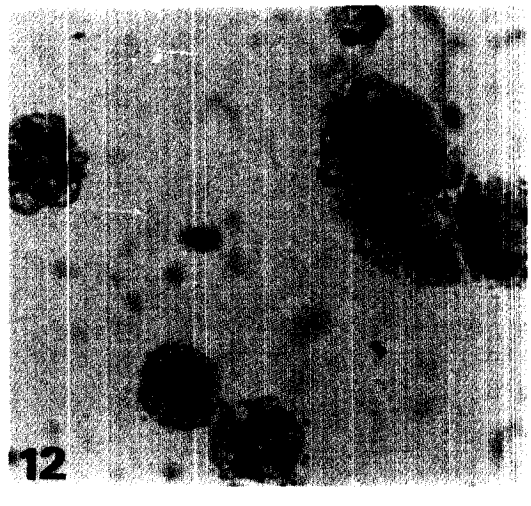

12

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