CHAPTER THREE

EFFECT OF INTRAPERITONEAL INJECTION OF IRRITANTS AND NON-SPECIFIC STIMULATION OF IMMUNE RESPONSE ON *EIMERIA NECATRIX* INFECTION.

INTRODUCTION

Intraperitoneal (i.p.) injection of irritants has been used widely as a method for obtaining large numbers of blood leucocytes (Simpson and Ross, 1971; Mahmoud, Warren and Boros, 1973) and macrophages (Sabet, Hsia, Stanisz, El-domeiri and Allen, 1977; Trifonov, Stoirov and Filchex, 1977; Long and Rose, 1976; Chvapil, Sakova, Bartos, Cox and Nichols, 1979; Sharma, Piessens and Middlebrook, 1980). Proteose peptone, casein, hydrolysed starch, Sephadex particles, thioglycolate broth and glycogen were used in these studies. Proteose peptone, starch and Sephadex G-50 particles have been used to obtain macrophages from the peritoneal cavity of birds (Long and Rose, 1976; Trifonov *et al.*, 1977; Sabet *et al.*, 1977). None of these workers used the i.p. injection of these irritants to modify leucocyte behaviour or to study their effects on an infection. Intraperitoneal injection of silica has, however, been used to inhibit adjuvant arthritis in rats (Bramm, Binderup and Arrigoni-Martelli, 1980).

Nonspecific stimulation with agents such as BCG has been used to enhance the sluggish or ineffective immunity frequently seen in malignant diseases (Keller, 1977; Campell, Sholley and Miller, 1980; Palladino and Thorbecke, 1977; Sharma *et al.*, 1980) and in parasitic infections (Weintraub and Weinbaum, 1977; Cox, 1978; Clark, Wills, Richmond and Allison, 1977; Clark, Allison and Cox, 1976; Smikovski

and Larson, 1977; Dolan, Brown and Cunningham, 1980). Bacillus Calmette Guerin (BCG), a strain of Mycobacterium bovis and Propionibacterium acnes (Corynebacterium parvum) have been used widely to stimulate nonspecific immunity against protozoal infections (Cox, 1978). The most dramatic protective effect of BCG was obtained in infections with Babesia spp. of rodents (Clark et al., 1976; Clark et al., 1977). Mice have been also protected to some extent against Plasmodium spp. when inoculated with BCG (Clark et al., 1976). BCG has also been shown to protect mice against Leishmania tropica and L. donovani (Weintraub & Weinbaum, 1977; Smrkovski and Larson, 1977) but inconsistent results have been obtained with Trypanosoma cruzi (Kierszenbaum, 1975; Brener and Cardoso, 1976). On the other hand BCG has failed to protect calves against Babesia divergens (Brocklesby & Purnell, 1977) and Theileria parva infection (Dolan et al., 1980). P. acnes inoculation has been shown to protect mice against Babesia spp. and Plasmodium spp. (Clark et al., 1977), T. cruzi (Kierszenbaum, 1975; Brener and Cardosa, 1976) and against infection with avirulent Toxoplasma gondii (Schwartzberg, Krahelbuhl and Remington, 1975).

In the work to be reported here, i.p. injection of irritants was initially used in an attempt to redirect leucocytes away from the site of infection in the small intestine. The hypothesis was, that heterophils were at least partially responsible for the lesions associated with E. necatrix infections and that by redirecting these cells into the peritoneal cavity the lesions could be modified. However, as will be seen from the results of these experiments an i.p. injection did not alter the intestinal lesions but instead interfered with the normal sequence of events in the life cycle of the

parasite. This was indicated by a delay in the appearance of first generation schizonts and lesions associated with the development of second generation schizonts and of peak oocyst production. As such, these irritants were later used to investigate the role played by the i.p. injection in modifying the life cycle of the parasite.

Similarly, BCG and P. acnes were used to explore their effects on the severity of lesions associated with the development of second generation schizonts, as well as their possible ability to protect chickens against E. necatrix infection. Results showed that the injection of BCG and P. acnes increased lesion severity and had no protective value against E. necatrix infection.

MATERIALS AND METHODS

1. Preparation and injection of irritants

A 10% solution of proteose peptone* in normal saline was autoclaved at 121°C for 10-15 minutes before use. One percent sterile Sephadex** G-75 suspension and 2% hydrolysed starch prepared in sterile normal saline and sterile thioglycolate* broth were also used as intraperitoneal irritants. The ideal i.p. injection site was the lower left side of the chicken body, 15-20 mm lateral to the keel bone and 30-40 mm above the cloacal pore. Seven to 15 ml of the appropriate irritant was used, depending on the size and age of the chickens. In

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* Difco Laboratories, Detroit 1, Michigan
** Pharmacia Fine Chemicals, Uppsala, Sweden

all experiments, irritants were injected i.p. except one in which proteose peptone was inoculated intramuscularly. Unless otherwise stated, injections were given at 0, 24, 48, 58, 59, 60, 72 or 96 hours post infection depending on the purpose of the experiment.

2. Preparation of Propionibacterium acnes vaccine

The strain used was kindly supplied by Dr. B.N. Wilkie, Department of Veterinary Microbiology & Immunology, OVC. The seed was first inoculated into tubes containing thioglycolate broth and incubated at 37°C for 2 days. The primary culture was then subinoculated into 3 litres of thioglycolate culture medium and incubated at 37°C for 3 days. The micro-organisms were killed by adding 0.5% formalin and incubating overnight at 37°C. Bacterial cells were recovered by centrifugation at 1500 xg for 15 minutes. They were washed three times with distilled water and freeze dried before use.

3. Bacilli Calmette Guerin (BCG)

BCG was obtained as live freeze dried vaccine* and was administered i.v. in 100 ug doses per chicken, as described in experimental design.

4. Toxic silica

Silica** was suspended in normal saline with the aid of a Vortex mixer, sterilized by autoclaving at 121°C for 10 minutes and

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* Connaught Laboratories Ltd., Willowdale, Ontario.
** Siliva was obtained through the courtesy of Steinkohlenberg-Bauverein. A.G. 43 Essen, W. Germany.

administered to chickens as soon as possible after preparation.

A general overview of experimental design is shown on page 118.

5. Effect of irritant injections on lesion severity and blood leucocytes

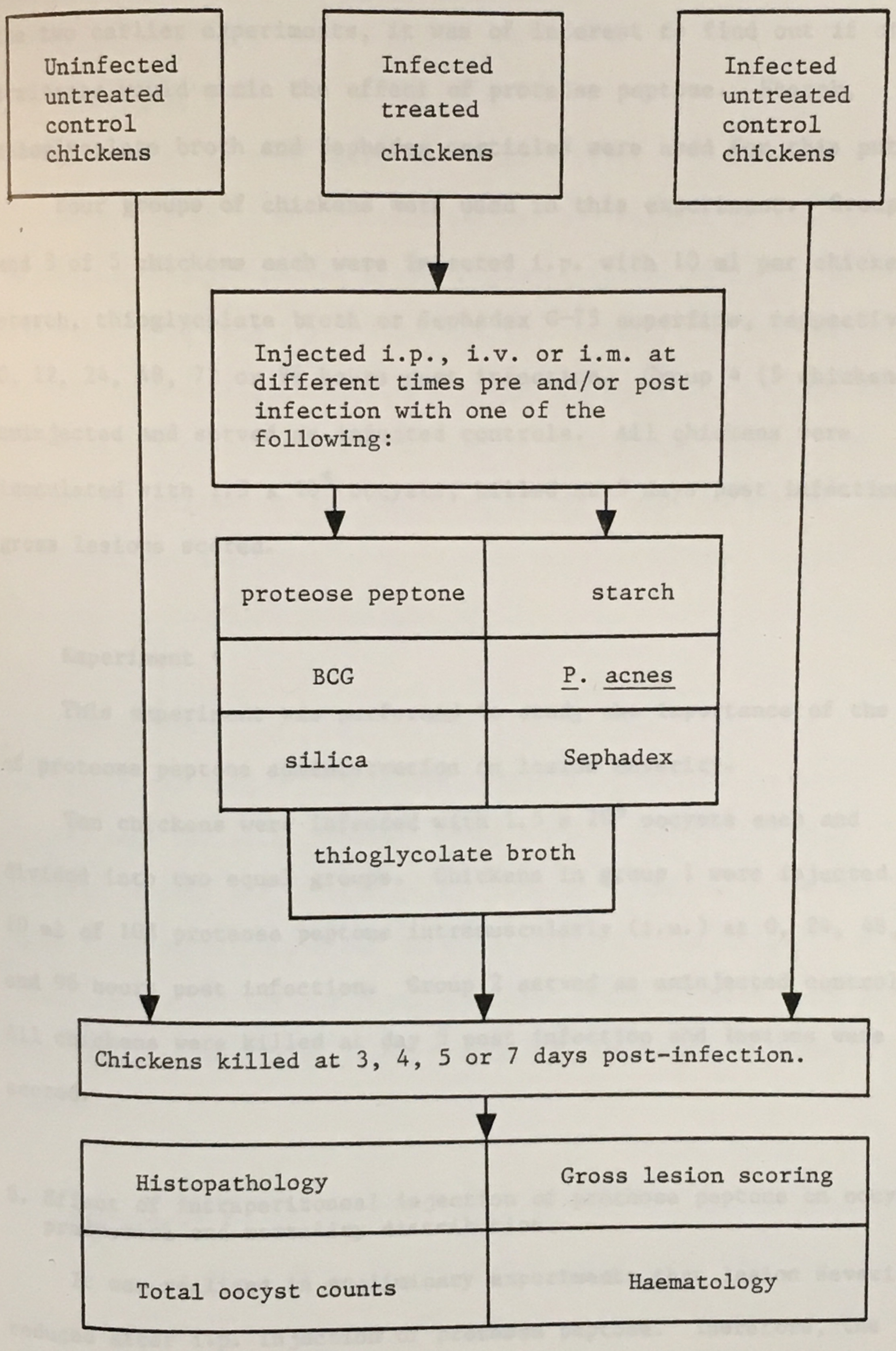
Experiment 1

Two groups of chickens were used. Group 1 had 7 chickens and each received i.p. injections of 10 ml of proteose peptone at 0, 24, 48, 72 and 96 hours post infection. Group 2 had 5 chickens and served as uninjected controls. Both groups were inoculated orally, each with 1.4×10^5 sporulated oocysts of E. necatrix in a 2 ml volume. Total blood leucocyte and differential counts were performed as detailed in Chapter 1. Chickens were bled at 0, 24, 48, 72, 96 and 120 hours post infection. All chickens were killed after the last bleeding and lesions scored as described in Chapter 1. Intestinal tissue and bone marrow samples were also taken for histological examination.

Experiment 2

Three groups of chickens were used. Group 1 of 4 chickens and group 2 of 3 chickens were injected i.p. with 10 ml of proteose peptone per chicken, at 0 and 12 hours post infection respectively and then both groups were injected every 24 hours up to day 4 post infection. Group 3 of 4 chickens served as uninjected controls. All groups were infected with 1.4×10^5 oocysts/chicken. Chickens were sacrificed and lesions scored at 5 days post infection.

Experimental Design



Experiment 3

As proteose peptone was used exclusively for the i.p. injection in the two earlier experiments, it was of interest to find out if other irritants would mimic the effect of proteose peptone. Starch, thioglycolate broth and Sephadex particles were used for this purpose.

Four groups of chickens were used in this experiment. Group 1, 2, and 3 of 5 chickens each were injected i.p. with 10 ml per chicken of starch, thioglycolate broth or Sephadex G-75 superfine, respectively at 0, 12, 24, 48, 72 or 96 hours post infection. Group 4 (5 chickens) were uninjected and served as infected controls. All chickens were inoculated with 1.5×10^5 oocysts, killed at 5 days post infection and gross lesions scored.

Experiment 4

This experiment was performed to study the importance of the route of proteose peptone administration on lesion severity.

Ten chickens were infected with 1.5×10^5 oocysts each and divided into two equal groups. Chickens in group 1 were injected with 10 ml of 10% proteose peptone intramuscularly (i.m.) at 0, 24, 48, 72 and 96 hours post infection. Group 2 served as uninjected controls. All chickens were killed at day 5 post infection and lesions were scored.

6. Effect of intraperitoneal injection of proteose peptone on oocyst production and mortality distribution.

It was realized in preliminary experiments that lesion severity was reduced after i.p. injection of proteose peptone. Therefore, the following experiments were conducted to determine if i.p. injection of

proteose peptone resulted in killing of the parasite, which would be expressed by reduction in daily oocyst output.

Experiment 1

Two groups of 5 chickens were used. Group 1 received one i.p. injection of 15 ml proteose peptone per chicken at time of infection. Group 2 served as uninjected infected control. Both groups were inoculated with 3×10^4 oocysts per chicken. At the beginning of the 6th day post infection each chicken was placed in a clean disposable cardboard box to collected feces for 24 hour oocyst counts. The first sample was obtained at the beginning of day 7 post infection. Oocyst production was monitored up to 13 days post infection. Oocyst counts were made as detailed in Chapter One.

Experiment 2

Eight chickens were infected with 1×10^3 oocysts each and divided into two equal groups. Group 1 was injected i.p. with 10 ml proteose peptone per chicken at the time of infection. Chickens in group 2 served as uninjected infected controls. One chicken was placed in each cardboard box and feces collected every 24 hours up to day 14 post infection and daily oocyst output was determined as indicated in Chapter One.

Experiment 3

As it was found from the preceeding experiments that the i.p. injection of proteose peptone resulted in a lengthening of the life cycle, this experiment was performed to determine if the i.p. injection

would delay the expected mortality seen in E. necatrix infections.

Eighteen chickens were infected with 1 x 10^5 oocysts and divided into two equal groups. Group 1 was injected with 10 ml proteose peptone i.p. and group 2 served as uninjected controls. Mortalities were followed in the two groups for up to day 9 post infection.

7. Effect of intraperitoneal injection of proteose peptone on the development of first and second generation schizonts.

Experiment 1

As it was found that second generation schizonts were retarded at 5 days post infection as a result of i.p. injection of proteose peptone, the following experiment was designed to determine if they resume normal development after day 5 post infection.

Two groups of chickens were used. Group 1 had 10 chickens which were injected i.p. with 10 ml proteose peptone each. Group 2 had 5 chickens which did not receive an i.p. injection. All chickens were inoculated with 1.2 x 10^5 oocysts each. Control chickens and 5 chickens from those that received the i.p. injection were sacrificed at day 5 post infection. The other half of the proteose peptone injected group was sacrificed at 7 days post infection and lesions were scored in all chickens.

Experiment 2

This experiment was done to determine what stage of schizogony was affected by the i.p. injection and whether or not an injection of proteose peptone at 24, 48 hours post infection had any effect on developing first generation schizonts.

Four groups of 5 chickens each were used. Chickens in group 1 were each injected with 12 ml proteose peptone at the time of infection, those in group 2 were injected at 24 hours and those in group 3 injected at 48 hours post infection. Chickens in group 4 were not injected with proteose peptone. All chickens were inoculated with 1.4×10^5 oocysts and sacrificed at 72 hours post infection. Intestinal samples were taken from areas 10, 11 and 12. The numbers of first and second generation schizonts in 100 crypt villous units were counted in each area (total 300 crypt villous units per bird).

Experiment 3

Part I - The results of the previous experiment indicated that the effect of the i.p. injection was on the early part of the life cycle of E. necatrix. Therefore the following experiments were designed to elucidate the early part of the life cycle, for example the route taken by the sporozoites to gain entry into the crypt epithelial cells, and to determine the effect of an i.p. injection on this process.

Two groups of chickens were used in this experiment. Twelve chickens of group 1 were injected with 10 ml of proteose peptone i.p. Twelve chickens of group 2 served as uninjected infected controls. All chickens were inoculated with 4.2×10^7 oocysts each. Four chickens from group 1 and 3 from group 2 were sacrificed at each time period of 6, 12 and 18 hours post infection. In each chicken the whole intestine was fixed in Bouin's fixative except for area 12 which was fixed in glutaraldehyde/paraformaldehyde and processed for electron microscopy as detailed in Chapter 1. Intestinal sections from areas

10, 11 and 13 were stained for the detection of sporozoites (Appendix I).

Part II - Two groups of chickens were used. Group 1 of 4 chickens were injected i.p. with 10 ml proteose peptone at the time of infection. Group 2 of 4 chickens served as uninjected controls. Chickens in both groups were inoculated with 12 million oocysts each. Two chickens from each group were sacrificed at 3 and 24 hours post infection. Intestinal tissues were processed for light and electron microscopy as described in part one of this experiment.

Experiment 4

Macrophages have been incriminated in the transport of E. necatrix sporozoites from villous to crypt epithelial cells. In addition the previous experiments suggested that the cell(s) involved in the transport of sporozoites may be attracted to the peritoneal cavity as a result of proteose peptone injection. Silica particles are known to kill macrophages selectively, therefore it was used to confirm that the cells involved in the transport of sporozoites were not macrophages.

Twenty-eight chickens were infected with 1×10^5 oocysts per ~~ded~~ chicken and divided into 4 equal groups. Group 1 was injected ~~ose~~ i.p. with 10 ml of 10% proteose peptone per chicken. Group 2 was injected i.p. with 400 mg of silica/chicken suspended in 10 ml of normal saline and group 3 injected with the same quantity of silica suspended in 10 ml of 10% proteose peptone. All i.p. injections were given at the time of infection. Group 4 served as uninjected infected control. All chickens were killed and lesions scored at 5 days post infection.

8. Effect of BCG and Propionibacterium acnes injection on lesion severity

Experiment 1

Seven chickens were divided into two groups of 3 and 4 chickens each. Those in group 2 were injected i.p. with 10 mg of P. acnes suspended in 5 ml sterile PBS at time of infection. The three chickens in group 1 were used as uninjected infected controls. All were inoculated with 1 x 10^5 oocysts each, sacrificed at day 5 post infection and lesions scored.

Experiment 2

Fourteen chickens were divided into 4 groups. Groups 1, 2 and 3 of 3 chickens each were injected i.p. with 20 mg of P. acnes suspended in 5 ml sterile PBS at 2, 1 and 0 days before infection respectively. The five chickens in group 4 were used as controls and were injected i.p. with 5 ml sterile PBS 24 hours before infection. All chickens were infected with 1.5 x 10^5 oocysts each, sacrificed at 5 days post infection and the lesions scored.

Experiment 3

Sixty chickens were divided into 11 groups. Groups 1, 2 and 3 had 5 chickens each and were injected intravenously (i.v.) with 100 µg of BCG vaccine suspended in 0.5 ml sterile PBS 14, 7 and 0 days before infection respectively. Groups 4, 5, 6, 7 and 8 had 5 chickens each. Group 4, 5 and 6 were injected i.v. with 1 mg of P. acnes suspended in 0.5 ml sterile PBS 14, 7 and 0 days before infection respectively. Groups 7 and 8 were injected i.p. with 2 mg of P. acnes suspended in