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COMPARISON OF RESISTANCE LEVEL AND CIRCULATING IgG RESPONSE IN CHICKENS EXPERIMENTALLY INOCULATED WITH A MULTIPLE OR A SINGLE IMMUNIZING DOSES OF *EIMERIA ACERVULINA*

M. M. GALMES*, C. C. NORTON**, J. CATCHPOLE**

SUMMARY

Two immunizing methods (Trickle or single immunizing doses) against *E. acervulina* were tested in chickens. The effects of immunization and challenge upon growth, oocyst output and circulating antibodies response (IgG) were compared. Neither immunization method produced pathogenic effects, similar numbers of oocysts were produced, and the levels of IgG in serum were similar and low in each case. After the challenge, immunized birds showed a high level of resistance but susceptible controls produced very large number of oocysts and showed a marked reduction in the growth. Birds immunized by a trikle infection produced oocysts on two days only and the total number of oocysts per bird was very low, whereas those immunized by a single infection produced oocysts over a period of nine days and the total number of oocysts was higher. Susceptible and birds immunized by a single inoculation showed similar IgG concentration and these were statistically higher than birds immunized by a trickle infection. In susceptible birds the kinetic of IgG was delayed about 4 days.

RÉSUMÉ : **Comparaison du degré de la résistance et du taux de IgG circulant chez des poulets inoculés avec une dose unique ou multiple d'oocystes d'*Eimeria acervulina*.**

On a comparé deux méthodes d'immunisation (dose unique ou multiple) des poulets vis-à-vis d'une infection par *E. acervulina,* en étudiant leurs effets sur le poids vif, l'excrétion oocystale et le taux d'anticorps circulants (IgG).

Aucune des deux méthodes d'immunisation n'a eu d'effet négatif sur le poids des animaux. L'excrétion oocystale et le taux de IgG dans le sérum ont été faibles dans les deux cas.

Après la réinfection, les animaux immunisés ont eu une croissance journalière égale à celle des témoins non immunisés-non infectés et statistiquement supérieure à celle des témoins infectés. Ces derniers ont éliminé un plus grand nombre d'oocystes.

Le taux de IgG est le même dans le sérum des témoins infectés et dans celui des poulets immunisés par une dose unique. Il est statistiquement plus faible chez les poulets immunisés par des doses multiples. D'autre part la cinétique des IgG chez les témoins infectés est retardée de 4 jours par rapport aux animaux immunisés.

INTRODUCTION

Circulating antibodies to *Eimeria* infections in chicken have been demonstrated by a number of tests in wich both particulate and soluble antigens have been used (Rose, 1982).

The ELISA test is suitable for detection of antibodies prepared from different developmental stages of *Eimeria* s.p.p. (Rose and Mockett, 1983 ; Saatara *et al.*, 1984 ; Mockett and Rose, 1986), and is more sensitive than IHA and IFA tests.

The present study was undertaken to compare a single and a trickle immunization with 20,000 oocysts of *E. acervulina,* analysing the effect of these infections and a subsequent challenge infection, on the growth of the birds, oocysts output and circulating IgG response.

* Departamento de Patología y Producción Animales, Facultad de Veterinaria, Universidad Autónoma de Barcelona, Cerdañola del Vallés, Barcelona, España.
** MAFF, Central Veterinary Laboratory, Weybridge, Surrey KT 15 3NB, U.K.

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MATERIALS AND METHODS

BIRDS

Day-old male light hybrid chicks (Ross Rangers) were obtained from a commercial hatchery and reared coccidia-free in wire-floored brooders in isolation until they were used in the experiment at seven days old (Day 0 of the experiment). No anticoccidial or antibiotic compounds were present in the food.

PARASITES

The Weybridge strain of *E. acervulina* was used. Birds were infected by inoculating oocysts directly into the crop.

Oocysts were 10 days old at Day 0. A fresh suspension was prepared for the challenge infection, and these oocysts were 10 days old at Day 21.

EXPERIMENTAL DESIGN

Chicks in Group A were dosed with 1,000 oocysts for 20 days.

Those in group B received 20,000 oocysts in a single dose given on Day 0, and chicks of Group C were non-infected controls.

Each of these groups included 7 replicates of 5 birds.

Groups A and B, and a susceptible control Group D containing 5 replicates of 5 birds each, were challenged on Day 21 with 1,000,000 oocysts.

Growth measurement

All chicks were weighed individually on Days 0, 3 to 10 inclusive, 15, 21, 24 to 30 inclusive, 37 and 42.

Faecal samples

The total faecal output was collected from each replicate daily from Day 5 to Day 42, and the oocyst output per bird per day was estimated by standard methods (MAFF, 1986).

Serum samples

Serum samples were obtained from clotted blood taken by wing vein puncture at different times after immunization and challenge (on alternate days from 7 to 21, 24 to 36, and Day 42). The serum samples were centrifuged before storage at −20° C.

Antigens

Soluble antigens were prepared from sporulated oocysts of *E. acervulina* as described previously by Rose (1977). Protein determination was carried out by the method of Lowry *et al.* (1951).

ELISA test

These were carried out as described by Voller *et al.*, 1976, using reaction volumes of 100 µl in microtitre plates (Dynatech Laboratories). The antigen, antiserum and conjugate were optimised before use. All plates contained positive and negative reference sera. Antigenes were diluted in carbonate buffer pH 9.6, to contain 150 µg protein ml.

The plates were incubated overnight at 4° C and then washed with PBS pH 7.2 containing Tween 20 (PBST). Any remaining non-specific sites were blocked by incubation for 45 minutes at 41° C in PBST containing 3 % bovine serum albumin (BSA, Sigma, London Chemical Co). This diluent, and these incubation conditions were used for subsequent steps.

Serum was diluted in 3 fold steps from 1/30 to 1/21,870.

Goat globulin raised against chick Fc portions of IgG (Nordic Immunological Laboratories) was used at a dilution of 1/100. Peroxidase conjugated rabbit anti-goat IgG (H + L) was used at a dilution of 1/300. The substrate used was 5-aminosalycilic acid and incubation was for 30 minutes at room temperature. The plates were washed three times between each step. Absorbance values were read on an automatic micro-ELISA reader (Dynatech Laboratories).

Statistical analysis

Live weight and IgG level were subjected to analysis of variance (Steel and Torrie, 1960) and group treatment means into each day compared by Duncan test.

RESULTS

Growth

The effects of the immunizing and challenge infections on live weight are given in *Table I* Chicks showed no retar-

Table I. — *Average of live weight per bird and treatment (grams).*

		Treatments					
Day (s)	*No. occysts administered* *No. of birds/treatment*	*A* *20 × 1,000*	*B* *20,000*	*C* *0*	*D* *0*	*SEM (1)*	*Significance (2)*
0	35	84.57	84.71	84.43		0.82	NS
3	35	115.14	113.97	113.37		1.49	NS
4	35	125.01	122.46	124.06		1.65	NS
5	35	133.83	129.91	132.71		1.77	NS
6	35	145.91	141.40	144.89		1.97	NS
7	33	157.18	151.97	156.91		2.23	NS
8	33	172.61	166.48	173.55		2.44	NS
9	33	184.06	177.94	183.73		2.66	NS
10	30	203.17	196.30	202.07		3.03	NS
15	28	271.68	269.19	266.79		4.26	NS
		(3)	(3)		(4)		
21	25	380.00	376.79	376.80	383.72	5.73	NS
24	18	439.83	432.47	437.83	428.06	8.72	NS
25	15	463.87(a)(5)	463.50(a)	466.13(a)	431.27(b)	8.5	*
26	15	484.13(a)	479.57(a)	507.40(a)	432.07(b)	9.85	***
27	15	513.13(a)	505.50(a)	516.07(a)	459.00(b)	9.32	***
28	13	526.46(a)	523.50(a)	530.69(a)	473.54(b)	10.03	**
29	13	549.00(a)	541.33(a)	554.85(a)	494.00(b)	10.35	***
30	13	571.23(a)	568.17(a)	574.92(a)	512.31(b)	10.5	***
31	10	591.40(a)	607.60(a)	601.60(a)	532.90(b)	13.18	**
37	10	732.90(a)	743.00(a)	744.70(a)	688.50(b)	14.84	*
42	10	858.50	872.90	872.00	813.90	18.1	NS

(1) SEM : standard error mean; (2) NS : not significant; * : $P < 0.05$; ** : $P < 0.01$; *** $P < 0.001$; (3) Challenge on day 21 (1,000,000 oocysts/bird); (4) Infection on day 21 (1,000,000 oocyst/bird); (5) Values in a horizontal line showing different superscripts are significantly different.

dation of growth during immunization. After challenge there were no significant differences between immunized Groups A and B and the non-infected control, Group C. In the susceptible birds, Group D, however, the daily mean weight decreased slightly on Day 25 *(Table I,* $P < 0.05$) and this became marked on Days 26 to 31 *(Table I,* $P < 0.01$ or $P < 0.001$). Weight gains improved on Day 37, but remained significantly below that of the other groups *(Table I* $P < 0.05$).

There were no significant differences among the four groups at the end of the experiment.

Oocyst production

The pattern of oocyst output of the different groups of birds during the immunization and challenge periods in given in *Table II.*

During immunization, birds which received a single dose produced nearly the same number of oocysts (20×10^6 per bird) as those which received 1,000 oocysts daily for 20 days (21.8×10^6 per bird). The bulk of oocysts from the single-dosed group appeared on Day 5, whereas the output from trickle-infected birds spread over several days and the period of patency was extended by one day.

After challenge, susceptible chicks in Group D produced 484 million oocysts per bird. Those which had received a single immunizing dose (Group B) produced 19 million oocysts per bird during a patent period of 9 days, whilst Group A, which had received a trickle infection, produced only 22 thousand oocysts and patent period was reduced to 2 days *(Table II).*

Antibodies in serum

The results of assay for the presence of specific IgG in serum are shown in *Figure 1.*

The points represent the mean values of 25 birds from Days 7 to 21; 20 birds for Day 24; 15 birds for Days 26 and 28; 13 birds for Day 30 and 10 birds thereafter.

Specific IgG was not detected during the immunization period except on Days 17, 19 and 21, when small amounts were recorded in a few birds only *(Fig. 1).* After chal-

Table II. — *The oocyst production (thousands of oocysts per bird) in groups of one-week-old chicks inoculated with 1,000 oocysts of Eimeria acervulina daily for 20 days (A) or a single of 20,000 oocysts on day 0 (B) and after challenge with 1,000,000 oocysts of Eimeria acervulina on day 21.*

Treatment *Infective dose (day 0)* *Number of oocysts* *Day (s)*	*A* *1,000 oocysts × 20*	*B* *20,000 oocysts*	*C* –	*D* –
5	1,413	12,883	–	
6	5,495	2,344	–	
7	6,457	3,311	–	
8	3,981	776	–	
9	2,339	490	–	
10	1,380	129	–	
11	537	46	–	
12	107	17	–	
13	20	1	–	
14	+	+	–	
15	+	2	–	
16-25	–	–	–	
Total × 10^6/bird	21.80	20.00		
	Challenge 10^6 oocysts on day 21			Infection 10^6 occysts on day 21
26	22	13,490	–	263,026
27	+	3,981	–	70,795
28	–	1,820	–	66,069
29	–	+	–	61,660
30	–	+	–	18,197
31	–	+	–	2,630
32	–	+	–	2,344
33	–	+	–	159
34	–	+	–	39
35	–	–	–	+
36	–	–	–	+
37-42	–	–	–	–
Total × 10^6/bird	0.02	19.00		

(1) Oocysts demonstrated by salt flotation concentration method, insufficient to count; (2) Oocysts no demonstrated by salt flotation concentration method.

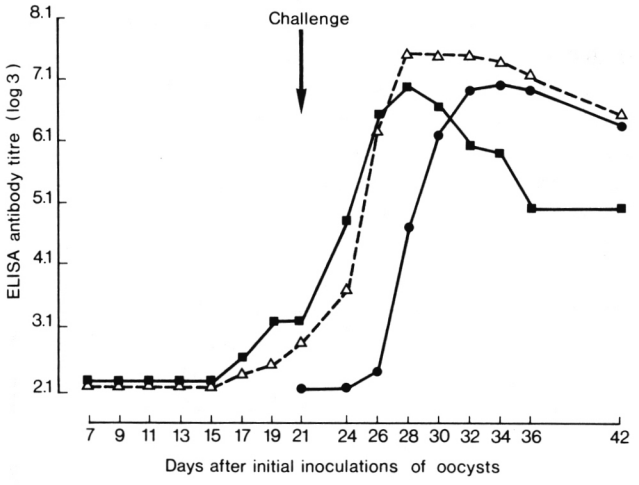

FIG. 1. — Sequential development of specific IgG antibodies to *E. acervulina* in serum samples from chickens of Group A, trickle infection of 20 × 1,000 oocysts (■), Group B, single infection of 20,000 oocysts (▲), and Group D, susceptible control to challenge infection of 1 million oocysts administered on Day 21 (●).

lenge, the IgG antibody level increased rapidly to reach its highest concentration on Days 28 (7 days after challenge) and then slowly declined in Group B and more rapidly in Group A. In susceptible controls (Group D), this profile was delayed and IgG antibodies were first detected on Day 26, reaching their maximum 6 days later *(Fig. 1)*.

The sera of non-infected chicks (Group C) remained negative immunoglobulins throughout the experiment.

In order to establish if there was any relationship among treatments and end point values for IgG, an analysis of variance was carried out on the $\log_3$ of the reciprocal end point titre. The results are given in *Table III*.

Immunized chicks showed similar concentration of IgG on Days 17 to 28. However there were significant differences in the value for IgG on Days 19 and 24 ($P < 0.05$). On Days 30 to the end of experiment chicks immunized by a single infection showed values statistically higher than chicks immunized by a trickle infection ($P < 0.05$ or $P < 0.01$) *(Table III)*. Susceptible chicks showed a lower concentration for IgG on Days 26 to 30 than chicks immunized by a single infection, however there were no significant differences between both treatment groups on Days 32 to the end of the experiment *(Table III)*.

DISCUSSION

The inoculation of 20,000 oocysts of *E. acervulina* to 7 day-old chicks, administered either as a single dose or by 20 daily doses of 1,000 oocysts produced no pathogenic effects *(Table I)*, whilst the total number of oocysts produced per bird was similar in each case *(Table II)*.

A very high level of resistance to reinfection was conferred by both multiple and single infections. After the immunized birds were challenged on day 21, their liveweight gain remained similar to that of non-infected controls *(Table I)*. Immunized birds passed only a few oocysts, but susceptible controls produced very large numbers *(Table II)*

TABLE III. — *Kinetics of serum IgG response in group A, trickle infection of 20 × 1,000 oocysts, group B, single infection of 20,000 oocysts and group D, susceptible control to challenge infection of 1 million oocysts administered on day 21.*

Treatment Day (s)	*A No. animals detected ×ELISA antibody No. animals tested*	*Titre $\log_3$*	*B No. animals detected ×ELISA antibody/ No. animals tested*	*Titre $\log_3$*	*D No. animals detected ×ELISA antibody/ No. animals tested*	*Titre $\log_3$*	*SEM (1)*	*Significance (2)*
7	0/25	2.1	0/25	2.1	—	—		—
9	0/25	2.1	0/25	2.1	—	—		—
11	0/25	2.1	0/25	2.1	—	—		—
13	0/25	2.1	0/25	2.1	—	—		—
15	0/25	2.1	0/25	2.1	—	—		—
17	4/25	2.5	2/25	2.3	—	—	0.1729	NS (3)
19	10/25	3.1(b)(4)	4/25	2.42(a)	—	—	0.2317	*(3)
21(ch.)(5)	12/25	3.2	7/25	2.74	0/25	—	0.2639	NS(3)
24(3)	16/20	4.7(b)	11/20	3.60(a)	0/20	—	0.3363	*(3)
26(5)	15/15	6.4(b)	14/14	6.2(b)	2/15	2.2(a)	0.2182	***
28(7)	15/15	6.9(b)	14/14	7.4(b)	13/15	4.6(a)	0.3207	***
30(9)	13/13	6.5(a)	12/12	7.4(b)	13/13	6.1(a)	0.2161	***
32(11)	10/10	5.9(a)	10/10	7.4(b)	10/10	6.7(a,b)	0.3109	**
34(13)	10/10	5.8(a)	10/10	7.3(b)	10/10	6.8(a,b)	0.3432	*
36(15)	10/10	4.9(a)	10/10	7.1(b)	10/10	6.7(b)	0.2981	***
42(21)	10/10	5.0(a)	10/10	6.4(b)	10/10	6.4(b)	0.2795	***

(1) SEM : standard error mean; (2) NS : not significant; * : $P < 0.05$; ** : $P < 0.01$; *** : $P < 0.001$; (3) Statistics analysis between groups A and B; (4) Values in a horizontal line showing different superscripts are significantly different; (5) Groups A, B and D challenged on day 21 (1,000,000 oocysts/bird).

and the birds showed a marked reduction in growth *(Table I)*. There were some differences in the level of resistance conferred by a single or multiple immunizing doses. Birds which received the trickle infection produced oocysts on two days only and the total number of oocysts per bird was very low, whereas those challenged after a single inoculum produced oocysts over a period of nine days and the total number of oocysts per bird was nine hundred times more *(Table II)*.

Trickle infections have been shown to confer a stronger and longer lasting immunity than a single inoculum containing the same number of oocysts (Joyner & Norton, 1976). On this occasion the phenomenon was supported by the difference in the duration of patency and by the total number of oocysts produced.

Serum IgG was first detected 17 to 19 days after immunization. The levels were generally low, and not all birds became positive. This may have been due to the low dose of 20,000 oocysts of *E. acervulina,* a species which is poorly immunogenic. Kuil *et al.*, (1977) and Saatara *et al.,* (1984) have shown that antibody response is directly related to the number of oocysts inoculated.

After challenge immunized chicks showed IgG response which was more rapid and more intense than primary response *(Fig. 1)*. Susceptible chicks had similar IgG response to chicks immunized by a single infection. There was a slight difference however in the kinetic of IgG between both treatment groups. Chicks immunized by a single infection showed a high concentration as early as 5 days after challenge, whereas in the susceptible chicks, peak concentration was delayed by about 4 days *(Fig. 1)*. It seems that the use of 20,000 oocysts of *E. acervulina* administered as a single dose produced a lower antigenic stimulus to determine the antibody response on the second contact with antigen to be more intense. Our results agree with those of Mockett and Rose (1986) indicated the serum IgG and the biliary IgA responses reappeared after the second and third inocula but there was no convincing evidence of anamnesis since the kinetics were largely similar to those found in the primary response.

The concentrations for IgG in the sera of chicks immunized by a trickle infection were significantly below that chicks immunized by a single infection. This may have been due to the degree of immunity produced by the mode of immunization. Rose and Mockett (1983) and Tanielian *et al.,* (1976) showed that challenge inoculations given after the establishment of complete immunity do not usually result in increased concentrations of antibodies. On this occasion, chicks immunized by a trickle infection showed a higher level of resistance than chicks immunized by a single inoculation since the last group produced a higher number of oocysts and had a prolonged patent period.

The present results demonstrate that the IgG antibody class appear following oocysts infection but it seems their value in protection is restricted since the pattern of IgG was similar between susceptible chicks and those immunized by a single infection. However they were susceptible and resistant, respectively, to challenge with *E. acervulina,* as judged by weight loss and reproduction of the parasite. Mockett and Rose (1986) indicated that immunity to Coccidiosis is T-cell dependent and cell mediated immune responses are probably of paramount importance, antibodies do participate in protection per se and, possibly, as modulators of cellular responses.

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