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Immune Responses in Infections with Coccidia: Macrophage Activity

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Peritoneal exudate cells from chickens immunized with two species of coccidia, *Eimeria tenella* or *Eimeria maxima*, were examined for their capacity to phagocytose stages of the parasite in vitro. True phagocytosis of the sporozoite stage is difficult to estimate because of its ability to invade cells, but may be evaluated by comparison with control suspensions. Peak activity (compared with cells from coccidia-free chickens) was found 3 to 5 weeks after the first inoulum of oocysts of *E. tenella*, and 1 week after the first inoculum of *E. maxima*— times which correspond to the onset of complete immunity to infection. Cells from coccidia-free chickens, in the presence of serum from birds immunized with *E. tenella*, phagocytosed sporozoites of *E. tenella* in a similar manner to cells from immunized birds. The immune serum had both cytophilic and opsonic adherence properties and the latter was species specific (for the two species tested).

Marcrophages appear to be involved in acquired immunity to the intracellular protozoa *Besnoitia* and *Toxoplasma* (17), but their role in infection with the closely related and economically important *Eimeria*, although suggested in 1959 (3), has been but little investigated. Huff and Clark (9) determined the numbers of sporozoites of *Eimeria tenella* within macrophages after injection into the coelomic cavity of normal or completely immunized chickens. Their finding of fewer sporozoites in the cells of immune birds was interpreted as indicating that the ability of macrophages from these birds to destroy the parasite was enhanced. Patton (16), also working with *E. tenella*, investigated the phagocytic activity in vitro of blood macrophages for sporocysts, and found greater numbers within cells from infected animals. The differences between these two reports may have been due to the different stages of the parasite used; the sporozoite may be more susceptible to intracellular digestion and, unlike the sporocyst, is invasive and capable of entering and leaving cells.

Further work on macrophage activity in infections with *E. tenella* and *Eimeria maxima* involving observations on both stages and an investigation of the role of antibodies is described below.

MATERIALS AND METHODS

Parasite. The Houghton strains of *E. tenella* and *E. maxima* were used for the greater part of the work but, for immunization with *E. tenella*, the initial infection was with an embryo-adapted strain in order to obtain a good immune response with the least pathogenic effect (12). The oocysts were produced and maintained by the standard methods of the laboratory, and sporozoites were obtained as previously described (13).

Experimental animals. HPRS Light Sussex chickens, 3 or 4 weeks of age at the beginning of the experiment, were kept free from coccidia until given oocysts.

Immunization. (i) E. tenella. Three inoculations each of 50,000 oocysts of *E. tenella* (the first one being the embryo-adapted strain) were given at 2-week intervals starting when the birds were 3 or 4 weeks old. The birds did not become completely immune and oocysts were present in the feces after the last inoculum, but they were protected from clinical coccidiosis, except in the first experiment where there was some blood in the feces after the second inoculum.

(ii) E. maxima. Two inocula of the more immunogenic species, *E. maxima*, were given. The first consisted of 1,000 oocysts at 3 weeks of age, and this resulted in complete immunity (no oocysts in the feces) to the second inoculum of 5,000 oocysts at 5 weeks of age.

Cell suspensions. Peritoneal cells (PEC) were harvested from chickens 24 to 48 h after the intraperitoneal injection of 5 to 10 ml of 3% hydrolyzed starch in saline, by washing out with 199 medium containing heparin (2 U/ml), penicillin, and streptomycin (40,000 U and 60 mg, respectively, per 100 ml) (M. E. Rose and P. Hesketh, Avian Pathol., in press). This medium, without heparin, was used for all other procedures. The predominant cell type appeared to be a macrophage; it was large, rounded with irregular edges, and contained a large nucleus and cytoplasmic granules which stained with neutral red.

Phagocytosis experiments. For simplicity, the term "phagocytosis" will be used although it is acknowledged that the presence of a sporozoite within a cell is not necessarily due to phagocytosis by the cell since sporozoites will invade (and leave) macrophages readily (6).

To overcome the difficulties involved in the identification of partly digested sporozoites within cells, the numbers of sporozoites remaining free in suspension after incubation with the various cell preparations were estimated, in preference to the numbers of cells containing parasites, or those to which parasites were attached.

Changes, resulting from infection, in the phagocytic activity of PEC were determined. Cells were collected at fortnightly intervals from three immunized and three control birds on each occasion, starting 1 week (week 1) after the initial oocyst inoculum. Suspensions were adjusted to a concentration of 1.2×10^6 per ml and added to an equal volume (usually 0.1 ml) of sporozoites, concentration 0.6×10^6 per ml, in small agglutination tubes. After incubation for 1 h at 41 C, the tubes were plunged into an ice-water bath and the numbers of free sporozoites in each tube were determined by using a hemocytometer; each test was replicated eight times. The percentage of reduction in numbers of sporozoites after incubation with a cell suspension gives the phagocytic activity (PA) of the cell suspension. The PA of control cells was determined by comparison with suspensions of sporozoites incubated with medium only; that of cells from immunized birds was determined by comparison with suspensions of sporozoites incubated with normal cells.

$$PA = \frac{\text{Concentration of sporozoites in control suspension} - \text{concentration of sporozoites in test suspension}}{\text{Concentration of sporozoites in control suspension}} \times 100$$

The significance of differences between the numbers of sporozoites present in the control and test suspensions was determined by using Student's t test.

Sensitization of cells or sporozoites. In experiments intended to determine the role of cytophilic or of opsonic adherence antibodies, cells from coccidia-free birds were used, henceforth called normal cells. Equal volumes of cell (2.4×10^6 per ml) or sporozoite (1.2×10^6 per ml) suspensions were incubated with heated (56 C for 30 min) normal or immune serum at 41 C for 30 to 60 min, centrifuged, and washed once in medium, and the concentrations were adjusted to the original value before use in phagocytosis tests as above.

In some experiments the serum dilutions were incorporated in the test suspensions so that the reactions took place in the presence of free antibody.

Tests in chambers. For observation and photographic recording of the parasite-cell interactions, the tests described above were also carried out in small chambers made from plastic rings, internal diameter 15 mm and 2 mm deep, fixed to cover glasses by silicone grease. The method was similar to that described for macrophage-erythrocyte interactions (2). Approximately 0.4 ml of the cell suspension was pipetted into the chambers and the cells allowed to attach for 30 to 60 min at room temperature. The suspension fluid containing unattached cells was then siphoned out and replaced with a similar volume of sporozoite suspension. The chambers were loosely covered and incubated at room temperature or at 41 C for 1 h. The chambers were emptied, thoroughly washed several times with medium to remove unattached parasites, and finally filled with a dilute solution of neutral red, and the chamber was sealed with a microscope slide. The macrophages on the under surface of the cover glass could then be examined. For permanent preparations the cover glasses were fixed in 5% formalin and stained with toluidine blue.

Cells could also be sensitized within the chambers by pipetting in the appropriate serum dilutions and incubating for 30 min at room temperature, after which they were thoroughly washed before addition of the sporozoite suspensions.

RESULTS

Sporozoite suspensions usually contained some sporocysts and when the numbers of sporocysts were sufficiently high to justify doing so, i.e., 10,000 per ml or greater, observations were made on these stages also.

Phagocytosis by normal cells. The degree of phagocytosis of sporozoites by cells from coccidia-free animals varied from sample to sample and from week to week, but was generally low. For *E. tenella* the mean PA value for 58 samples was 4.1%. The data obtained in experiments 1, 2, and 7 (below) were analyzed and the effects of normal cells on the numbers of sporozoites was found to be not significant.

For sporozoites of *E. maxima*, an analysis of the data obtained from experiment 3 (below) showed the effects to be significant ($P < 0.05$), not differing significantly from week to week; the average reduction was 9%.

The values for sporocysts were higher; for *E. tenella* (30 samples), the range was 0 to 84% with a mean value of 39%, and for *E. maxima* (18 samples), the range was 0 to 54% with a mean value of 24%.

Thus, phagocytosis of sporozoites of either species by normal cells was low, but the numbers of *E. maxima* were reduced to a somewhat greater extent than those of *E. tenella* after incubation with normal cells. For sporocysts, phagocytosis by the same cell samples was considerably higher, but the numbers available for examination were low in many cases.

Phagocytosis by immune cells. The phago-

cytic activities of cells from immunized animals were compared with those from controls at fortnightly intervals throughout each of two similar experiments (experiments 1 and 2) with *E. tenella* and one (experiment 3) with *E. maxima*. The results obtained for sporozoites are shown in Fig. 1 and 2, in which the PA of immune cells compared with normal cells is plotted against time. Three immunized birds and three controls were sampled at each time interval.

In the experiments with *E. tenella* (Fig. 1), a definite pattern emerged; phagocytic activity increased from week 1 after oocyst inoculation to reach a peak at week 5 and then declined. A fourth inoculum of oocysts given after the three immunizing infections was followed by an increase in PA 1 or 2 weeks later. Differences between weeks are significant ($P < 0.01$). More data on the changes in phagocytic activity of cells during immunization were obtained from experiment 7 (Fig. 3A); peak activity here was found 3 weeks after the initial inoculum and the PA reached zero at 9 weeks (no booster inoculum given). The differences between values are significant ($P < 0.01$). These results differed significantly ($P < 0.01$) from those found in experiments 1 and 2, possibly due to the 2 weeks interval between observations.

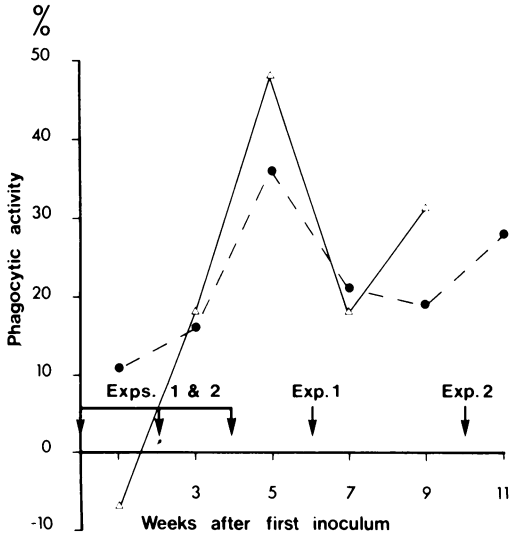

FIG. 1. *E. tenella. Phagocytic activity for sporozoites of E. tenella of cells removed from immunized birds at fortnightly intervals. Each point is a mean value for estimations on three immunized birds compared with three controls. The residual standard deviation of the log transformed data was 0.055. Symbols: Δ, experiment 1; ●, experiment 2; ↓, oocyst inoculum given.*

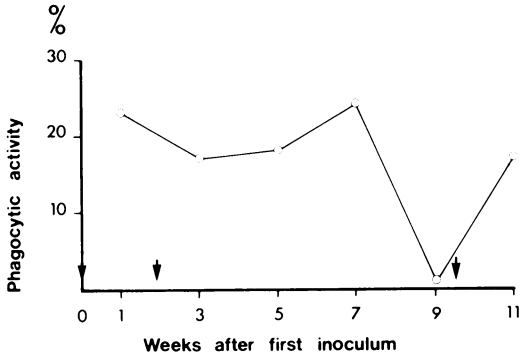

FIG. 2. *E. maxima. Phagocytic activity for sporozoites of E. maxima of cells removed from immunized birds at fortnightly intervals. Each point is a mean value for estimations on three immunized birds compared with three controls. The residual standard deviation of the log transformed data was 0.040. ↓, Oocyst inoculum given.*

In the experiment involving *E. maxima* (Fig. 2), a highly significant ($P < 0.001$) increase in PA was obtained 1 week after infection; this was maintained until week 7, and the effect did not differ significantly from week to week. The average PA was 16%. A challenge inoculum, to which the birds were immune, was followed by a rise in PA.

The results obtained with sporocysts of both species are incomplete but show a similar trend to those obtained with sporozoites, i.e., peak activity for both stages coincided. PA values for sporocysts were again higher than those for sporozoites.

Role of antibody. The possible role of antibody in the enhanced activity of macrophages from animals immunized with *E. tenella* was investigated by using normal cells and allowing the cell-parasite interactions to take place in the presence of serum, or by pretreating the cell (passive direct test) or sporozoite (passive indirect test) suspensions with serum which was washed off before the suspensions were mixed. Immune serum was obtained from pooled blood samples of chickens immunized as before and bled 4 weeks after the initial oocyst inoculation. This was the time at which the macrophages might be expected to exhibit peak activity (from experiments 1, 2, and 7). Normal serum was obtained from birds of a comparable age kept coccidia free.

The results of four experiments (4A–D) in which serum, normal or immune at a dilution of 1:30 was present in the reaction mixture, are shown in Table 1. Immune serum significantly increased phagocytosis of sporozoites by normal cells, and this was most marked with unheated

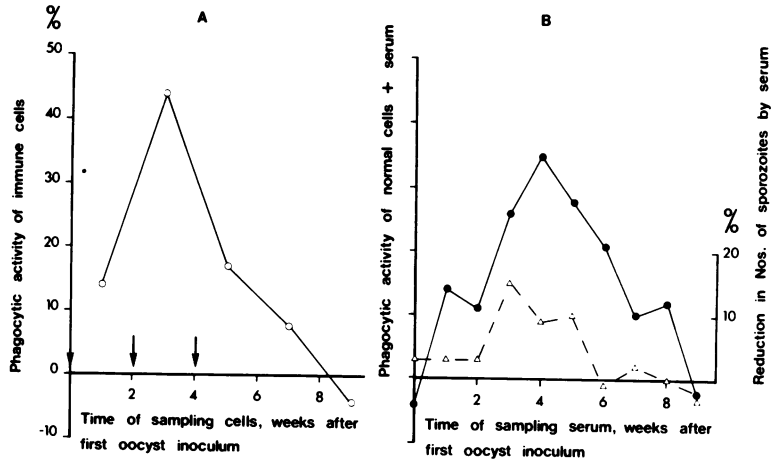

FIG. 3. *(A) Phagocytic activity of cells from immunized birds (immune cells) for sporozoites of E. tenella. Each point is a mean value for estimations on three immunized birds compared with three controls. Average normal "phagocytic" activity was 9%. ↓, Oocyst inoculum given. (B) Effect of serum samples from the same group of immunized birds on (i) the phagocytic activity of normal cells for sporozoites of E. tenella (●) and (ii) number of sporozoites of E. tenella after incubation in serum alone (△).*

TABLE 1. *Phagocytic activity of normal cells for sporozoites of E. tenella: effect of the presence of serum in the reaction mixture*

Expt no.	Reaction mixture added to sporozoite suspensions	Sporozoites present (no. × 10^3/ml)	Reduction (%)	Sporocysts present (no. × 10^3/ml)	Reduction (%)
4A	Medium	340		15	
	Medium + NC	326.7	4	12	20NS
	Normal serum	354		10.8	
	Normal serum + NC	285	19[a]	6	44[a]
	Immune serum	219.2		17	
	Immune serum + NC	100	54[a]	3.3	85[a]
	Heated normal serum	295		14.2	
	Heated normal serum + NC	289.2	2NS	2.5	77[b]
	Heated immune serum	263.3		13	
	Heated immune serum + NC	222.5	15[a]	1	92[a]
4B	Medium	257.5		5.8	
	Medium + NC	270	0	0	
	Immune serum	155		3.3	
	Immune serum + NC	50.8	68[a]	0	
	Heated immune serum	322.5		5.8	
	Heated immune serum + NC	152.5	53[a]	0.8	
4C	Medium	354.2		10	
	Medium + NC	370	0	6.7	
	Heated normal serum	346.7		7.5	
	Heated normal serum + NC	362.5	0	5.8	
	Heated immune serum	269.2		8	
	Heated immune serum + NC	245.8	9[c]	3.3	
4D	Medium	307.5		9.2	
	Medium + NC	286.7	7[c]	10	0
	Heated normal serum	321.7		14.2	
	Heated normal serum + NC	331.7	0	10	30NS
	Heated immune serum	279.2		14.2	
	Heated immune serum + NC	226.7	19[a]	4.2	70[b]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.02$; NS, not significant; NC, normal cells. All serum dilutions are 1:30.

serum. After heating, normal serum had little, if any, effect on the PA of normal macrophages but unheated normal serum caused a significant increase (experiment 4A). Because of this, and the deleterious effect of unheated immune serum on the sporozoites (compare suspensions a and c, experiment 4A and suspensions a and b, experiment 4B) all sera were heated in subsequent experiments. The highest dilution at which this heated immune serum enhanced the PA of normal cells was approximately 1:50.

Similar findings were obtained for sporocysts; the results have been worked out only in those experiments (4A and D) where the numbers in the control suspensions exceeded 10,000 per ml. As with other experiments, activity was greater with sporocysts than with sporozoites.

The effect of sensitizing normal cells with 1:30 serum is shown in Table 2 (experiments 5A-C). Treatment with heated immune serum enhanced the PA of normal cells in all three experiments; heated normal serum caused a significant increase (giving a value outside the normal range) only in experiment 5A. These results indicate that antibodies present in the serum of immunized animals became attached to normal macrophages and thereby enhanced their phagocytic activity for sporozoites and sporocysts.

In mixtures where sporozoites were sensitized with heated normal serum (experiment 6A-D) the PA of the cells was within the normal range, but sensitization with heated immune serum resulted in a greater uptake of sporozoites (Table 3).

To determine whether treatment of normal cells and sporozoites with serial samples obtained throughout immunization could produce a pattern of PA similar to that found with the cells of immunized birds, experiment 7 was set up. A group of birds was given three immunizing inocula (*E. tenella*) as before; throughout the experiment, three birds were killed at fortnightly intervals and the PA of their cells was determined (compared with cell suspensions from a similar number of control birds). The group was also bled at weekly intervals and the individual samples were pooled. Finally, the effect of adding the serial serum samples to portions of a suspension of normal cells was determined. Controls consisting of sporozoites plus serum samples without cells were included. All sera were inactivated before use and diluted to give a final concentration of 1:30. Results are presented in Fig. 3.

The pattern of rise and fall of PA of cells from infected birds during immunization was similar to that found previously except that the peak occurred at week 3 (week 4 untested).

Serum obtained at weeks 3 to 5, although inactivated and diluted, caused some reduction in the numbers of sporozoites ($P < 0.01$) (also seen in experiments 4A, C, and D). Serum samples taken at the other time intervals did not significantly affect sporozoite numbers. The PA of normal cells was unaffected by the presence of week 0 and week 9 serum samples but serum samples weeks 1 to 8 inclusive increased the PA of normal cells, the differences in numbers of sporozoites present in serum-cell mixtures being significantly ($P > 0.001$) different from those present in any of the control

TABLE 2. *Phagocytic activity of normal cells for sporozoites of E. tenella: effect of pretreatment of cells with serum before addition to parasite suspensions (passive direct test)*

Expt no.	Reaction mixture added to sporozoite suspensions	Sporozoites present (no. $\times 10^3$/ml)	Reduction (%) compared with		Sporocysts present (no. $\times 10^3$/ml)	Reduction (%) compared with	
			a	b		a	b
5A	a Medium	209.2			48.3		
	b Medium + NC	199.2	5NS		30.8	36[a]	
	Medium + NC(HN)	151.6	28[a]	24[a]	26.7	45[a]	13NS
	Medium + NC(HI)	103.3	51[a]	48[a]	14.2	71[a]	54[a]
5B	a Medium	291.3			0.6		
	b Medium + NC	267.5	8[a]		0		
	Medium + NC(HN)	261.9	10[a]	2NS	3.8		
	Medium + NC(HI)	241.3	17[a]	10[b]	1.3		
5C	a Medium	380			6.3		
	b Medium + NC	350	8[b]		3.8		
	Medium + NC(HN)	375.6	1NS	0	3.8		
	Medium + NC(HI)	305	20[a]	13[a]	1.3		

[a] $P < 0.001$; NC, normal cells; NC(HN), normal cells treated with heated normal serum 1:30; NC(HI), normal cells treated with heated immune serum 1:30; NS, not significant.

[b] $P < 0.01$.

TABLE 3. *Phagocytic activity of normal cells for sporozoites of E. tenella: effect of pretreatment of sporozoite suspensions with serum (passive indirect test)*

Expt no.	Reaction mixture	Sporozoites present (no. × 10^3/ml)	Reduction (%)	Sporocysts present (no. × 10^3/ml)	Reduction (%)
6A	Spzs(HN) + medium	296		20	
	Spzs(HN) + NC	326.7	0	23.3	0
	Spzs(HI) + medium	358.3		31.7	
	Spzs(HI) + NC	275.8	23[a]	17.5	45[a]
6B	Spzs(HN) + medium	366.9		5	
	Spzs(HN) + NC	326.9	11[a]	0.6	
	Spzs(HI) + medium	324.4		7.5	
	Spzs(HI) + NC	218.4	33[a]	0	
6C	Spzs(HN) + medium	380		8.8	
	Spzs(HN) + NC	339.3	11[a]	2.9	
	Spzs(HI) + medium	371.9		11.9	
	Spzs(HI) + NC	273.8	26[a]	1.9	84[a]
6D	Spzs(HN) + medium	383		11	
	Spzs(HN) + NC	385	0	5	55[b]
	Spzs(HI) + medium	391		18	
	Spzs(HI) + NC	368	6[b]	6	67[a]
	Spzs(HN) + medium	359.2		13.3	
	Spzs(HN) + NC	366.7	0	9.2	31[b]
	Spzs(HI) + medium	385		20.8	
	Spzs(HI) + NC	306.7	20[a]	4.2	80[a]

[a] $P < 0.001$; Spzs(HN), sporozoites treated with heated normal serum; Spzs(HI), sporozoites treated with heated immune serum; NC, normal cells. All serum dilutions 1:30.

[b] $P < 0.02$.

mixtures (serum alone, cells alone, medium alone). The variation in PA in mixtures of cells and serum with time of sampling was very similar to that obtained with cells (presumably sensitized in vivo) from infected birds, with peak values at weeks 3 to 5. Thus the presence of serum from infected birds caused the cells of normal birds to exhibit PA similar to that of the cells of the serum donors.

The specificity of the enhancing effect of immune serum on PA was tested by treating sporozoites of *E. maxima* with the *E. tenella* immune serum used in experiments 4 to 6, and vice versa. The *E. maxima* immune serum was a pool taken from a group of birds 14 days after they had received a single inoculum of 2,000 oocysts of *E. maxima;* it was highly protective (18). Results are given in Table 4. The sensitizing effect of *E. tenella* immune serum was species specific, treatment with it having no effect on the numbers of sporozoites of *E. maxima* in suspension. In the reverse situation, treatment with *E. maxima*-immune serum did cause a slight but not significant reduction in the numbers of *E. tenella* sporozoites in the presence of cells, whereas treatment of the homologous organisms resulted in a fairly small but significant increase in phagocytic activity.

Observations on cell-parasite interactions. The cell-parasite interactions were similar, irrespective of whether immune cells or passively sensitized normal cells (passive direct test) or sporozoites (passive indirect test) were used. In the sensitive systems, sporozoites (and other stages when present) became attached to the cells and were not washed away in the final stages of preparation (Fig. 4B). In the nonsensitive systems (normal cells, untreated sporozoites), very few parasite stages were seen, (Fig. 4A). Sporozoites were attached to cells end- or sideways-on, often more than one to a cell. Movement of attached sporozoites often continued, especially when the temperature was raised, e.g., on examination after storage at 4 C. In some instances they appeared as if trying to free themselves, which they sometimes did. In these cases a "bridge" connecting cell and parasite was sometimes visible and this became stretched until it seemed to give way (Fig. 4C–F and G). Sporozoites and other stages of the organism were also found inside the cells, usually after incubation at 41 C (Fig. 4H).

DISCUSSION

The tests for phagocytosis described here were simple to carry out, less time consuming, and probably more reliable than tests involving the preparation and examination of cell suspensions and the identification of possibly partly

TABLE 4. *Specificity of the opsonising activity of sera derived from birds immunized with E. tenella or E. maxima*

Reaction mixture[a]	Mean sporozoites present (no. × 10^3/ml)	Reduction (%)	Mean sporocysts present (no. × 10^3/ml)	Reduction (%)
E. tenella + medium	93.3		13.3	
E. tenella + NC	91.6	2NS	8.3	38[b]
E. tenella (Ets) + medium	276.6		15	
E. tenella (Ets) + NC	245	11[c]	15.8	0
E. tenella (Ems) + medium	310		20.8	
E. tenella (Ems) + NC	295	5NS	22.5	0
E. maxima + medium	247.5		8.3	
E. maxima + NC	259.2	0	17.5	
E. maxima (Ems) + medium	367.5		29.2	
E. maxima (Ems) + NC	333.3	9[d]	11.6	60[c]
E. maxima (Ets) + medium	417.5		13.3	
E. maxima (Ets) + NC	417.5	0	10	25NS

[a] *E. tenella* (Ets), sporozoites of *E. tenella* treated with serum from birds immunized with *E. tenella*; *E. tenella* (Ems), sporozoites of *E. tenella* treated with serum from birds immunized with *E. maxima*; *E. maxima* (Ems), sporozoites of *E. maxima* treated with serum from birds immunized with *E. maxima*; *E. maxima* (Ets), sporozoites of *E. maxima* treated with serum from birds immunized with *E. tenella*; NC, normal cells; NS, not significant.

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] $P < 0.001$.

digested parasites within cells. Although the majority of the data relates to the invasive stage, it is likely that the decrease in numbers of sporozoites observed in the sensitive systems reflects phagocytosis or adsorption by the cells, rather than invasion of cells by the parasite. Incubation with normal PE cells had little effect on the numbers of untreated sporozoites of *E. tenella* and an equilibrium of sporozoites entering and leaving cells is probably established. In a similar way, *Toxoplasma* organisms will leave normal macrophages and invade cultured mouse fibroblasts (17). It is possible that, when the numbers are decreased after incubation with normal PE cells, this is a reflection of the state of health of the sporozoite suspension. Normal sporozoites are fairly active and enter and leave cells very readily; those whose activity is impaired may be more likely to be phagocytosed by a cell and less able to escape. This may explain the greater reductions found with the more delicate sporozoites of *E. maxima*.

Cells from immunized animals caused significant reductions in the numbers of sporozoites in suspension. This supports the view (9) that the finding of fewer sporozoites within immune macrophages was probably due to increased intracellular digestion. In the case of *E. tenella*, peak activity was found 3 to 5 weeks after the first immunizing inoculum, probably corresponding fairly well to the immune status of the cell donors since *E. tenella* is a weakly immunogenic species requiring more than one infection to induce a high degree of immunity. An additional stimulus given in experiments 1 and 2 at 6 and 10 weeks, respectively, was followed by increased activity.

A single inoculum with *E. maxima* usually results in prompt and complete immunity (19) and, in the birds immunized with this species, the phagocytic activity of the PE cells had attained peak values by the time of the first testing, i.e., 1 week after the initial inoculum. This level was maintained until week 9, when it dropped almost to zero but returned to the previous immune level when the birds were reinfected.

The findings relating to sporocysts are very similar, although the data are incomplete. With this nonmotile stage the PA of cells, both normal and immune, was much higher than with sporozoites, but immune cells were more active than control cells, confirming the work on the interactions of sporocysts and blood macrophages (16). The sporocyst, although readily phagocytosed, may be more resistant to intracellular digestion than the sporozoite, and this may account for the differing previous reports.

Thus phagocytic activity as measured in the in vitro tests seems to correlate fairly well with the immune status of the cell donors.

The maximum values obtained were of the order of a 50% reduction in the numbers of

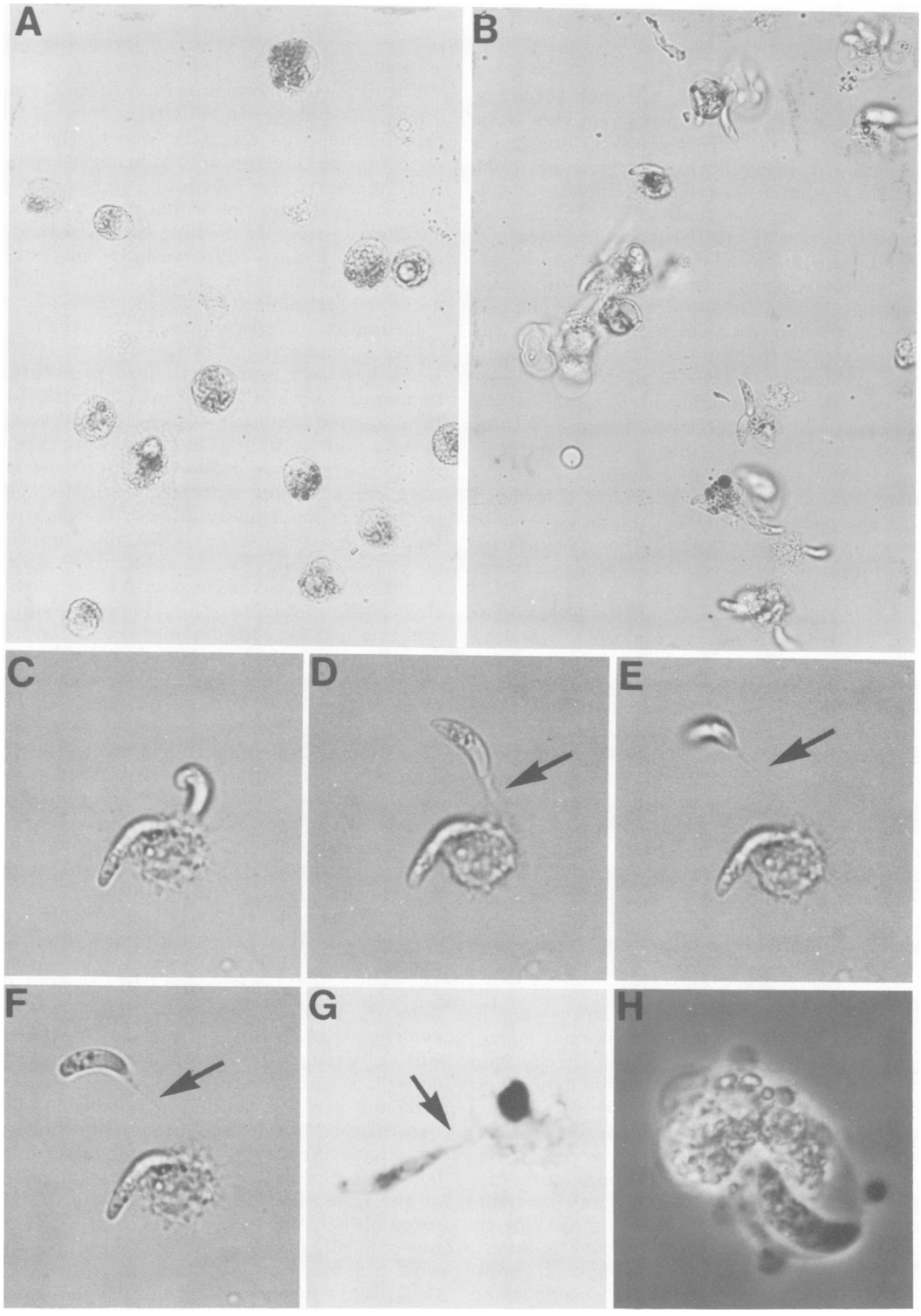

FIG. 4. *Interactions of peritoneal exudate cells with sporozoites of E. tenella. (A–F) Observations on cells in chambers. G fixed and stained (toluidine blue) preparation. (A) Control cells incubated (room temperature) with sporozoites treated with heated normal serum (diluted 1:15). No sporozoites attached (×540). (B) Control cells incubated (room temperature) with sporozoites treated with heated immune serum (diluted 1:15). Note attached sporozoites (×500). (C–F) As B. One sporozoite firmly attached, nonmotile; second sporozoite detaching itself from cell. Note "thread" of attachment (arrowed) (×1,000). (G) As A. Fixed and stained preparation. Note attachment of sporozoite (×2,000). (H) Control cell containing sporozoite treated with heated immune serum (diluted 1:30) (×1,880).*

sporozoites in suspension, although there was always an excess of cells over sporozoites in the reaction mixture. This is probably due to the formation of an equilibrium between adsorption and desorption as described by Rowley (21) in explanation for the findings of Auzins and Rowley (1) that about 50% of bacteria remained free in suspensions of macrophages and bacteria. Certainly, sporozoites were seen detaching themselves from the surfaces of macrophages after incubation at room temperature. It was also noticeable that, on first inspection of incubation chambers, very few if any free sporozoites were seen, unattached sporozoites having been removed during the washing process, but, on standing for some time, more became evident, presumably due to a reversal of attachment and/or phagocytosis. Rowley considered that reversal would be less likely in vivo due to trapping of bacteria against other cell surfaces, and presumably this could also apply to sporozoites.

Although there is some conflict, current opinions on the "immune macrophage" favors the view that its enhanced activity is due either to nonspecific maturation in response to the stimulus of the infection, or to the presence of a layer of cytophilic antibody on its surface, and there is, as yet, no evidence for the existence of a true specifically immune macrophage (see reference 4). All macrophages used in this study, whether from normal or immune animals, were "activated" in that they had been stimulated by hydrolyzed starch; consequently the differences observed were not due to nonspecific stimulation and maturation. They could have been due to the acquisition of a layer of cytophilic antibody by the macrophages. This was investigated by testing the effect of serum from infected birds. The presence of this serum in reaction mixtures conferred on normal macrophages the phagocytic activities of cells from immunized animals. The reduction in effect on heating (seen also with normal serum) suggests that heat labile factors are important for maximum phagocytosis (21).

The enhancing factors in immune serum could be demonstrated equally well by treatment of normal cells (passive direct) or by treatment of the parasites (passive indirect), followed in either case by washing before addition to the reaction mixtures. Whether one and the same antibody is responsible for both manifestations is not clear from the present work; it is possible that both macrophage cytophilic antibodies and opsonic adherence antibodies are present simultaneously (see 23).

Clearly, even normal serum caused some increase in phagocytic activity in some of the experiments; this might be due to the supplementation of factors present in normal serum and necessary for optimum phagocytosis, and/or to the presence of naturally occurring antibodies. It is also possible that a coating of serum, not removable by washing, may make parasites adhere more readily to the cells—a necessary preliminary for phagocytosis.

Incubation of sporozoites of *E. tenella* in heated immune serum in low dilution (1:3 or 1:1.5) has been reported to lead to eventual immobilization and loss of infectivity (8, 14). Incubation in heated immune serum (weeks 3 to 5) at a dilution of 1:30 in the present experiments caused some deleterious effects. Sporozoite numbers were reduced by 9 to 15% and morphology was slightly altered; the parasites tending to assume a more "stumpy" appearance. Also, after centrifugation, there was a tendency, especially in the case of *E. tenella*, for slight agglutination to occur. However, motility was retained for considerable periods; treated sporozoites attached to cells were motile after incubation at room temperature for 1 h followed by 18 h at 4 C.

Enhanced uptake of parasite stages by cells from immunized animals and by cells from normal animals after treatment of the cells and/or parasites with immune serum has thus been shown in vitro. Whether immune phagocytosis plays a role in immunity to infection is not known but it would be reasonable to assume that it could do so provided that the conditions were suitable. Obviously, contact between cells and parasite is a prerequisite; the intestinal epithelium is fairly well supplied with macrophages and, in the case of *E. tenella*, *E. necatrix*, and *E. acervulina*, there is some evidence that sporozoites may be transported within macrophages to their developmental sites (3, 5, 7, 15, 24); therefore, contact between cells and parasite could occur readily in vivo. Sensitization of cells and/or parasites could be by circulating or by locally produced antibodies; local vascular permeability is increased at the time of infection, especially in immunized birds (11, 20). Very little is known of the role of locally produced antibodies in infections with *Eimeria*, but there are indications that they may be involved (M. E. Rose, unpublished data).

In the in vitro work described here, no attempt was made to determine whether the viability of sporozoites was affected by reaction with sensitized macrophages. Cell adhesion was

seen to be a reversible process in a proportion of cases; sporozoites were seen attempting to, and sometimes succeeding in, disengaging themselves from macrophages. The effect of residence within a sensitized macrophage on subsequent infectivity deserves investigation. The staining characters alone of the parasites within the cells are probably not sufficiently reliable (17), but electron microscope studies of the host cell-parasite interactions would probably provide useful information (see 10). The presence of damaged merozoites within cells thought to be macrophages has been reported (22). Investigations on the infectivity of sporozoites after a period within immune macrophages would necessitate the use of methods capable of detecting very small differences in infectivity, since the greatest reduction in numbers of sporozoites seen in this study was of the order of 50%

The species specificity of the effects described here (also found in some of the immune macrophage-sporocyst combinations studied by W. H. Patton [personal communication]), paralleling the specificity of immunity in vivo, further suggests that phagocytosis may be one of the defence mechanisms involved in immunity to coccidial infections.

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