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Coccidiosis: Rapid Depletion of Circulating Lymphocytes After Challenge of Immune Chickens with Parasite Antigens

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Characteristic changes in the numbers of peripheral blood leukocytes occurred after specific challenge of chickens immunized by infection with the intracellular intestinal coccidian parasite *Eimeria maxima*. Within minutes of enteric or intravenous challenge with viable parasites or with soluble parasite antigen, the numbers of circulating lymphocytes and, to a lesser extent, of heterophils were reduced. This was followed by a period of leukocytosis, the main cellular constituents of which were heterophils and lymphocytes. Indirect fluorescent staining with antisera to T- or B-lymphocytes showed the depletion in lymphocytes to be accounted for mainly by a reduction in the number of T-cells. The leukopenia after oral challenge, found in immunized birds, could be transferred to normal birds by the intravenous injection of serum, plasma, extracts of leukocytes, or suspensions of viable spleen cells.

Coccidia of the genus *Eimeria* are intracellular protozoan parasites, mainly of the epithelial cells of the gut. A previous study (16) showed that in primary infections in rats and chickens there is a biphasic blood leukocytosis involving lymphocytes, polymorphonuclear neutrophils or heterophils, and large mononuclear cells. In immune chickens the response to challenge, examined from 3 h after inoculation, was accelerated and brief; it consisted of a single increase in lymphocytes and heterophils and was accompanied by cellular infiltration of the villi of the small intestine, which are the sites of invasion. The leukocyte response was specific to the species used for the primary infection and was not evoked by challenge with a closely related species which infects approximately the same site.

We have now reexamined the peripheral blood leukocyte (PBL) response in chickens, paying particular attention to events occurring within 3 h of challenge, a time period not previously investigated.

MATERIALS AND METHODS

Animals. The animals used were Light Sussex or inbred East Lansing Line 6 (White Leghorn type) male and female chickens of the Houghton Poultry Research Station strains and were kept free from specified pathogens until inoculated with *Eimeria maxima* at 3 or 4 weeks of age. Line 6 chickens were used only in the experiment in which viable spleen cells were transferred.

Parasites. Methods for handling the parasites, which were the Houghton strains of *E. maxima* and of *Eimeria acervulina*, and for counting oocysts (for dosing or for estimation of infection) have been fully described previously (7). Sporozoites were excysted in vitro (21) and either injected intravenously or inoculated into the duodenum after laparotomy (19).

Soluble parasite antigen. The soluble parasite antigen was prepared from suspensions of oocysts in phosphate-buffered saline (pH 7.0) by disruption with glass beads, followed by repeated freezing and thawing (12). The optical density at 280 nm was 24 U/ml.

Examinations of blood. Blood samples were taken, leukocytes were counted, and cells were differentiated from smears as previously described (16). Lymphocytes were differentiated into B- or T-cells by an indirect immunofluorescence test, using specific anti-T- or anti-B-cell serum prepared in rabbits and fluorescent-conjugated goat anti-rabbit globulin serum (9). When tested against suspensions of thymus or bursa cells, the anti-T- and anti-B-cell sera stained >95% of the homologous and <3% of the heterologous cell suspension.

Serum, plasma, and leukocyte extracts. The serum, plasma, and leukocyte extracts were prepared from the pooled normal and immune blood samples of groups of 15 birds. The immune materials were obtained from birds bled 21 days after oral inoculation with 3×10^3 oocysts. Control (normal) samples were obtained from uninfected birds of the same hatch. Blood was taken by venepuncture or cardiac puncture into two syringes, one of which contained heparin (final concentration, 5 to 10 U/ml) to serve as the source of plasma and leukocytes for the preparation of cell extract.

The heparinized blood was centrifuged at $60 \times g$ for 8 min, and the plasma containing undeposited leukocytes was removed. This suspension, consisting mainly of lymphocytes (>95%), was further centrifuged at $500 \times g$ for 8 min, and the plasma was removed and pooled. The deposited cells were washed twice and resuspended in medium 199 to the original volume of blood. They were then frozen and thawed three times and centrifuged at $500 \times g$ for 8 min, and the supernatant solution was removed and pooled. This pool was diluted 1/10 to provide a generous approximation of the cell products which might be released into serum as a consequence of damage during clotting.

Serum obtained by centrifugation from the clotted samples of unheparinized blood was pooled and, like plasma and cell extract, stored at −20°C before use. Three batches of immune and normal preparations were made and used in separate experiments.

Spleen cells. Spleens were obtained from Line 6 chickens 6 weeks after they had been inoculated with 3×10^3 oocysts of *E. maxima* and from uninoculated coccidia-free birds of the same hatch. Suspensions of cells, prepared as described before (14), were 93% viable by exclusion of trypan blue solution and consisted of lymphocytes (70%) with some large mononuclear cells (25%) and heterophils (5%).

Antibodies to soluble parasite antigen. Antibodies to soluble parasite antigen were detected by an enzyme-linked immunosorbent assay (20).

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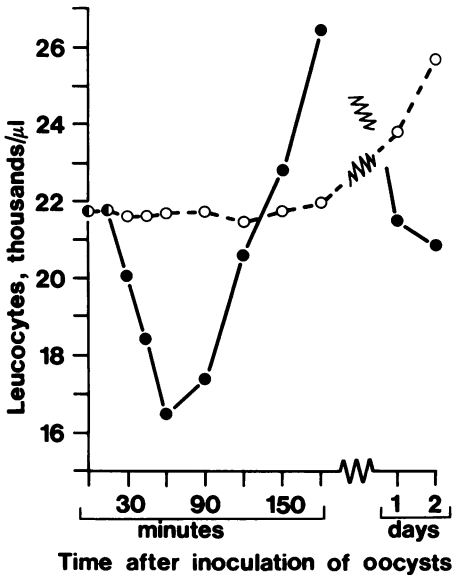

FIG. 1. Numbers of PBLs in previously coccidia-free (○) and immune (●) birds after oral challenge with 8×10^5 *E. maxima* oocysts. There were four birds per group. Mean values are plotted.

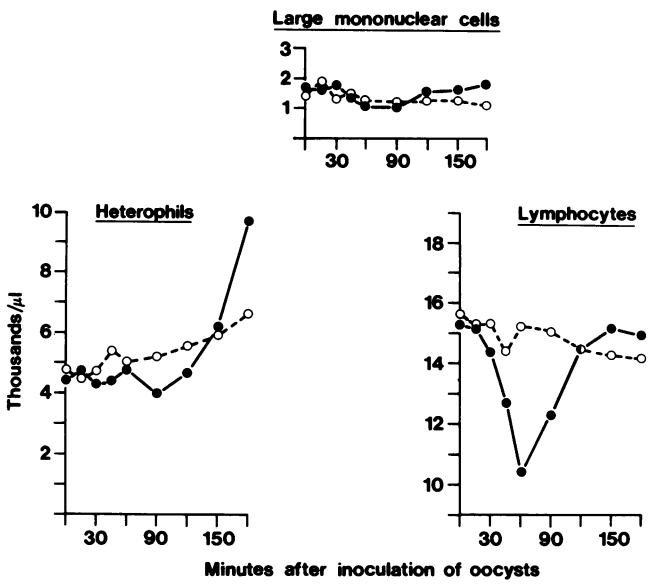

FIG. 2. Results of differential leukocyte counts on blood smears of birds whose total leukocyte counts are given in Fig. 1. Symbols: ○, previously uninfected birds; ●, immune birds.

Design of experiments. Chickens were immunized by the infection which resulted from the inoculation of 15×10^3 oocysts into the crop (immune chickens). Three weeks after dosing with oocysts, the birds were challenged with 8×10^5 oocysts via the crop, with an equivalent number of viable sporozoites intravenously or intraduodenally, or with 0.5 ml of soluble parasite antigen intraduodenally or intravenously. Blood samples were examined at frequent intervals on the day of challenge and at daily intervals thereafter. After challenge with viable parasites (oocysts or sporozoites), the feces were examined for oocysts to determine whether infection had occurred. In experiments in which serum, plasma, or cell extracts were transferred, coccidia-free, 6-week-old Light Sussex chickens were injected intravenously with serum, plasma, or cell extract (dose, 0.8 ml per 100 g of body weight) and challenged orally with oocysts 30 min later. Suspensions of viable spleen cells (6×10^8 per bird) were given intravenously to 4-week-old coccidia-free Line 6 chickens 7 days before challenge.

Each group contained 4 to 6 chickens; mean values are given in the figures, and the significance of any differences is given by a sign test. Analyses of variance were carried out on some of the data presented in tabular form.

RESULTS

PBL response to challenge with viable parasites or soluble parasite antigen. (i) After oral inoculation of oocysts. The numbers of PBLs in groups of immunized and normal chickens bled at short intervals after a challenge inoculation of 8×10^5 oocysts of *E. maxima* are shown in Fig. 1. In immunized birds there was an initial leukopenia (mean reduction, 23.7%; range, 15.4 to 27.3%) in the first hour, followed by a recovery to preinoculation values and then a leukocytosis (mean increase, 23%; range, 16 to 35%). There was little change in the numbers of leukocytes in the blood of previously uninfected birds during the initial period of testing, but they began to increase on day 1.

In immune birds the changes occurred in the numbers of heterophils and lymphocytes (Fig. 2). The initial leukopenia was due very largely to a depletion of lymphocytes (mean reduction, 27%; range, 15 to 47%), and, in the experiments shown in Fig. 2, the leukocytosis was largely due to an increase in the numbers of heterophils (mean, 11.7%; range, 75 to 170%), but in other experiments the numbers of lymphocytes were also increased at 180 min. The results obtained from blood smears made during the later period of

TABLE 1. Effect of challenge with *E. acervulina* on PBL counts in *E. maxima*-immune birds

Oocysts used for:		No. of PBLs ($10^3/\mu l$)[b]					
Immunization	Challenge[a]	Time (h) after inoculation of oocysts				Decline (0 to 1 h)	Recovery (1 to 7 h)
		0	1	2	7		
E. maxima	*E. maxima*	33.4	28.0	29.6	38.3	5.4[c]	10.3[d]
E. maxima	*E. acervulina*	29.7	30.7	29.1	27.1	−1.0	−3.6[e]
E. maxima	None	31.7	32.0	31.6	31.9	−0.3	−0.1

[a] 8×10^5 oocysts given.

[b] Analyses of variance to study the effects of challenge (in comparison with no challenge). Trends over time were examined by evaluating differences corresponding to the declining (0- to 1-h) and the recovery (1- to 7-h) phases. Significance tests were based on variations between birds (9 df). The significances of the effects of *E. maxima* challenge were $P > 0.05$ at 0, 1, and 2 h after inoculation; $0.01 < P < 0.05$ at 7 h after inoculation; $0.001 < P < 0.01$ at decline; and $P < 0.001$ at recovery. The significances of the effects of *E. acervulina* at 0, 1, 2, and 7 h after recovery, at decline, and at recovery were $P > 0.05$ (not significant). The standard errors were 1.74, 1.26, 1.50, and 1.91 for 0, 1, 2, and 7 h, respectively, after inoculation; 1.13 for decline; and 1.51 for recovery.

[c] $0.001 < P < 0.01$.

[d] $P < 0.001$.

[e] $0.01 < P < 0.05$.

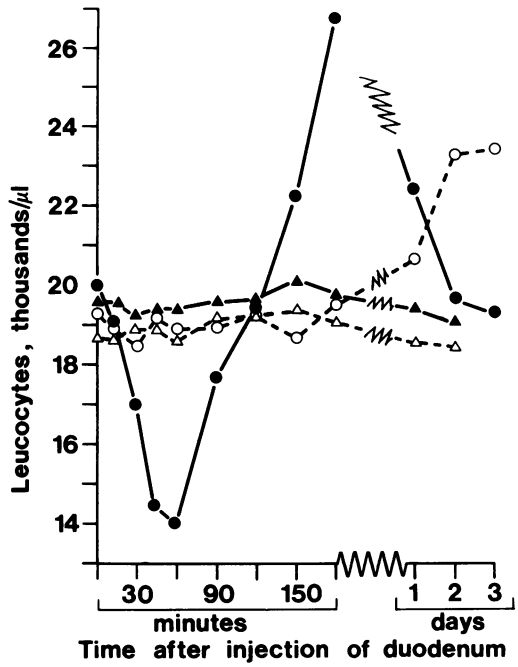

FIG. 3. Numbers of PBLs after intraduodenal inoculation of sporozoites (○, ●) or of saline (△, ▲). Closed and open symbols refer to immune and previously uninfected birds, respectively. There were four birds per group. Mean values are plotted.

testing indicated an increase in both lymphocytes and heterophils up to and including 7 h after dosing.

In nonimmune birds there were much smaller changes with no lymphopenia at 1 h, but there was a gradual increase in the numbers of heterophils from 0 to 3 h (mean, 35%; range, 12.7 to 56.9%).

The early leukocyte response was shown to be specific to the immunizing antigen by the results (Table 1) of challenge with oocysts of *E. acervulina*, a species which inhabits approximately the same area (proximal small intestine) of the gut as *E. maxima*. The effect of challenge with *E. acervulina* on the numbers of circulating leukocytes was not significant, apart from a slight suggestion of a decline from 1 to 7 h. In contrast, the effect of challenge with *E. maxima* was to produce a significant decline in the numbers of circulating leukocytes up to 1 h, followed by a significant increase to a level above that of controls at 7 h. In a separate experiment in which a control, unimmunized group was inoculated with *E. acervulina*, there were no changes during the first 3 h and a slight change at 7 h, which, as in the nonimmune birds mentioned above, included an increase in the numbers of heterophils.

A complete dose response study was not made, but, in one experiment, immune birds were challenged with 10^3, 10^4, or 10^5 oocysts and the percent increases in numbers of leukocytes at 7 h postinoculation were 16, 18, and 52, respectively. Thus the response was dose dependent but could be elicited with moderate numbers of oocysts.

(ii) After intraduodenal or intravenous inoculation of sporozoites. Some time must elapse between the inoculation of oocysts into the crop and contact of the host tissues with the invasive stages, the sporozoites. To determine with more accuracy the time interval between host-sporozoite contact and changes in numbers of leukocytes, 6×10^6 viable sporozoites (approximately equivalent to 8×10^5 oocysts given orally), excysted in vitro, were introduced directly into the duodenum. After intraduodenal inoculation, there was a drop in the numbers of PBLs when the birds were first sampled at 15 min (Fig. 3), whereas in the birds given oocysts orally, there was no change at 15 min (Fig. 1). However, the remainder of the response was similar, with maximum depression (mean, 32%; range, 28 to 35%) at 60 min, a return to normal by 2 h, and leukocytosis (mean increase, 29%; range, 20 to 43%) at 3 h. The numbers of PBLs in comparable birds, immune and control, did not

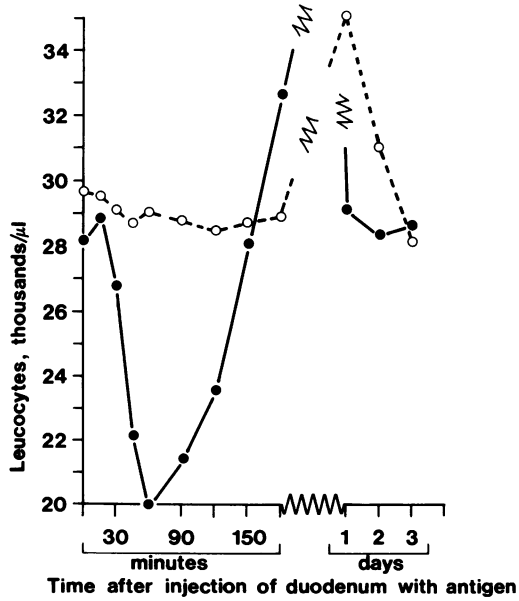

FIG. 4. Numbers of PBLs after intraduodenal inoculation of soluble parasite antigen into immune (●) or previously uninfected (○) birds. There were four birds per group. Mean values are plotted.

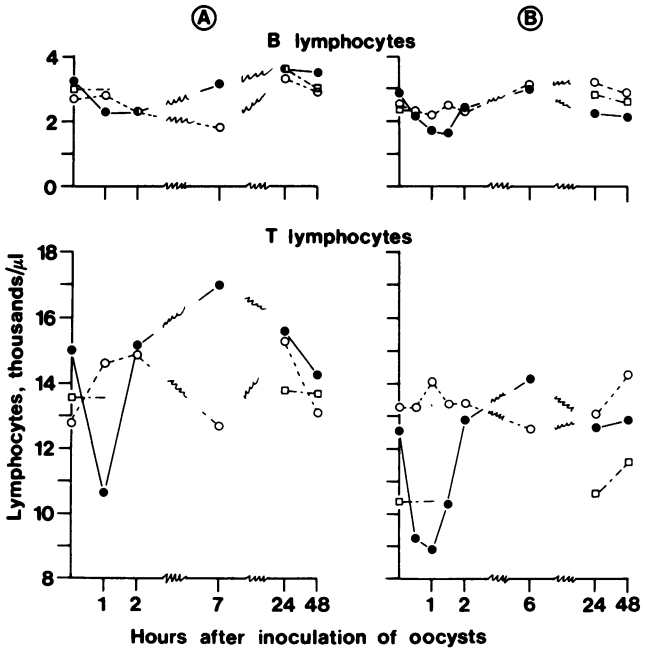

FIG. 5. Numbers of circulating T- or B-lymphocytes after oral challenge of immune (●) or previously uninfected (○) birds with 8×10^5 *E. maxima* oocysts and also in control, untreated birds (□). There were six birds per group. Mean values are plotted.

TABLE 2. Effect of challenge with *E. maxima* on PBL counts in recipients of serum, plasma, or cell extract

Recipient	Mean decline in no. of PBLs ($10^3/\mu l$) 0 to 1 h after challenge[a]						
	Immune donor			Normal donor			None
	Serum	Plasma	Cell extract	Serum	Plasma	Cell extract	
Challenged	6.1 (12)	2.8 (11)	4.2 (12)	−0.7 (4)	−1.3 (4)	−0.6 (1)	−0.2 (6)
Unchallenged	0.2 (5)	−2.2 (2)	0.1 (3)	Not tested			0.2 (6)

[a] *E. maxima* oocysts (8×10^5) were given 30 min after injection of serum, plasma, or cell extracts. The values within parentheses are the numbers with positive decline. $n = 12$ birds for all experiments, except $n = 8$ birds for the unchallenged recipient with immune donor.

change significantly after intraduodenal inoculations of similar volumes of phosphate-buffered saline.

The intravenous inoculation of 6×10^6 sporozoites caused changes (data not shown) in PBLs very similar to those found in birds given sporozoites intraduodenally. The feces of the nonimmunized birds contained numerous oocysts on day six after injection, indicating that infection of the gut followed intravenous inoculation of sporozoites; there was no infection in the previously infected birds.

(iii) After challenge with soluble parasite antigen. The PBL responses of immunized birds to antigen given intraduodenally (Fig. 4) or intravenously (data not shown) were the same as those which occurred after the administration of viable parasites; no changes in PBLs were seen during the initial test period in nonimmunized birds.

Thus, in birds immunized by infection, characteristic changes in the numbers of PBLs (heterophils and lymphocytes) occurred within minutes of enteric or intravenous challenge with either viable parasites or soluble parasite antigen. The significance of the decline during the first hour, and the ensuing recovery to preinoculation levels and leukocytosis, was obvious from the fact that the changes took place in each of the 12 birds (sign test, $P < 0.001$).

Initial lymphopenia after challenge involves mainly T-lymphocytes. In two experiments involving challenge with oocysts given orally, the circulating lymphocytes were differentiated according to their staining properties with specific antisera to B- or T-lymphocytes. The results of a preliminary experiment (Fig. 5A) indicated that the numbers of both B- and T-lymphocytes were reduced during the early part of the response of immune birds. In the second experiment, the birds were bled more frequently and the depletion of T- and B-lymphocytes was confirmed (Fig. 3B). However, the lymphopenia was largely accounted for by the T-lymphocytes. Changes in total leukocytes, total lymphocytes, and heterophils occurred as before and are not shown.

Transfer of response with serum, plasma, cell extracts, or suspensions of viable spleen cells. The results (data not shown) of preliminary experiments indicated that the response of immune birds to challenge, i.e., initial leukopenia followed by leukocytosis, could be transferred to normal recipients by the intravenous injection of serum from birds bled 14, 18, or 21 days after infection. No such changes were apparent in birds given normal serum and challenged or in challenged but otherwise untreated birds of the same hatch.

In three additional experiments the effects of serum, plasma, and leukocyte extract obtained 21 days after infection were compared. A different pool of donor birds was used for each experiment, but, within each experiment, all test materials were prepared from the same source of pooled blood. All three pools of serum and plasma samples from the convalescent birds contained small amounts of antibodies when tested by enzyme-linked immunosorbent assay ($\log_2$ titers, 5.6 to 7.3).

The averaged results of the three experiments are shown in Fig. 6, and the data are summarized statistically in Tables 2 and 3. The effect of handling the chickens to inject the reagents, assessed by comparing the numbers of leukocytes in blood samples taken immediately before injection with those in samples taken 30 min later, immediately before the inoculation of oocysts, was shown by analysis of variance to be not significant. In 35 of the 36 chickens given reagents from immune donors, challenge caused a depletion in the numbers of lymphocytes at 1 h, which was followed by recovery and leukocytosis at 6 or 7 h, a highly significant result ($P < 0.001$). The leukopenia was most marked in the chickens given serum (mean decrease, 26.2%) and least in the recipients of plasma (mean decrease, 12.7%). However, the plasma sample used in experiment 2 caused some leukocytosis in unchallenged animals, as did the control plasma sample in challenged chickens (Table 2), and, in both cases, the proportion of granulocytes appeared high. The results of a subsequent trial in which birds were injected with saline containing the equivalent amount of the heparin used to prepare plasma for experiment 2 indicated that this batch of heparin could cause some granulocytosis and that this may have masked the full extent of leukopenia.

The other treatments resulted in a slight increase (not significant) in the numbers of leukocytes over the period of the experiment.

The results (Table 4) show that leukopenia occurred 1 h after challenge in the recipients of viable spleen cells from

TABLE 3. Effect of challenge with *E. maxima* on PBL counts in recipients of serum, plasma, or cell extract

Recipient	Mean recoveries in no. of PBLs ($10^3/\mu l$) 1 to 7[b] h after challenge[a]						
	Immune donor			Normal donor			None
	Serum	Plasma	Cell extract	Serum	Plasma	Cell extract	
Challenged	12.1 (12)	8.8 (12)	7.6 (11)	0.2 (7)	0.7 (8)	0.1 (7)	0.8 (7)
Unchallenged	0.8 (4)	1.7 (5)	3.1 (7)	Not tested			0.4 (7)

[a] *E. maxima* oocysts (8×10^5) were given 30 min after injection of serum, plasma, or cell extract. The values within parentheses are the numbers with positive recovery. $n = 12$ birds for all experiments, except $n = 8$ birds for unchallenged recipient with immune donor.

[b] Six hours for experiment 1.

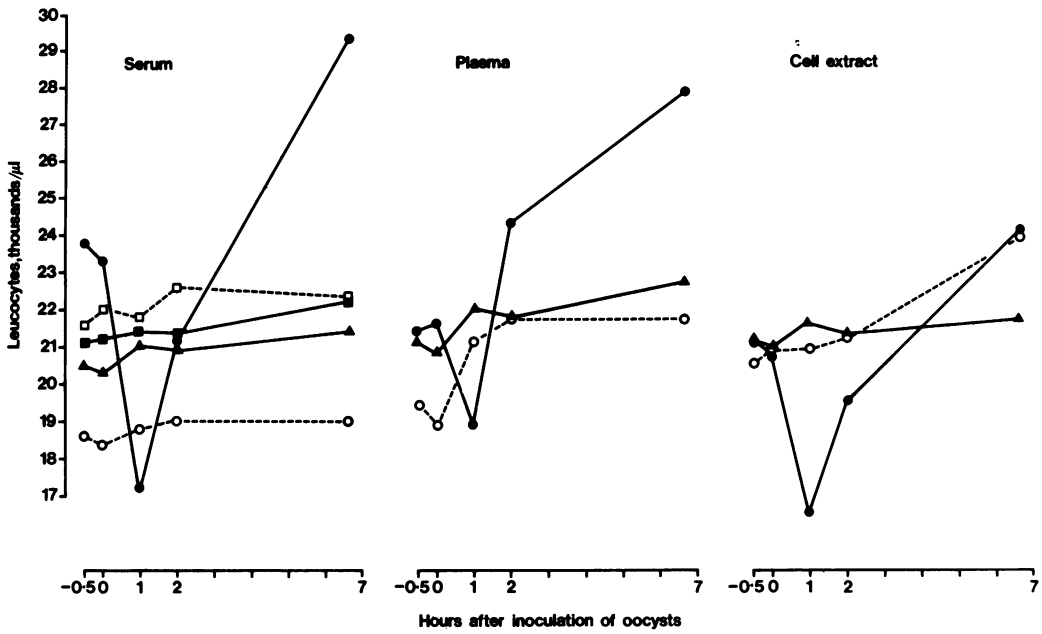

FIG. 6. Numbers of circulating leukocytes in recipients of serum, plasma, or cell extract. Serum, plasma, or cell extract from immune donor, recipients challenged (●), unchallenged (○); reagents from normal donor, recipients challenged (▲), challenge only (■); untreated (□). Mean values are plotted. There were 8 or 12 birds per group (Tables 2 and 3).

immunized birds, but there was no leukocytosis at 7 h. Initially, the recipients of spleen cells had significantly more circulating leukocytes than did the control group; the numbers in the latter remained fairly constant throughout the period of testing.

Thus the early lymphopenic response of immune birds to challenge inoculations with oocysts could be transferred to normal birds with serum, plasma, cell extracts, or viable suspensions of spleen cells, but the later response of leukocytosis was not consistently obtained.

DISCUSSION

Stimulation with viable parasites, or with soluble antigen, of previously immunized birds caused marked changes in the numbers of circulating leukocytes. In an earlier paper (16) which described a response occurring from 3 h after challenge with viable parasites, it was suggested that the changes in PBLs could represent mobilization of cells, possibly to the site of challenge, i.e., the gut, the walls of which were found to contain large numbers of lymphocytes and heterophils 7 h after dosing. The present findings indicate that changes in circulating leukocytes occur within minutes of challenge and are manifested by an initial removal of cells from the circulation. This was more evident with lymphocytes than with heterophils, but the numbers of the latter are probably very rapidly supplemented by the release of cells from the marginal pool (2, 10). Both B- and T-lymphocytes were affected, but, because of their greater numbers in the circulation, the depletion of T-lymphocytes was chiefly responsible for the lymphopenia. Thus challenge of immunized birds with the viable parasite, or its antigens, resulted in the mobilization of heterophils and of (mainly) T-lymphocytes via the circulation. These early circulatory changes appear to be immunologically mediated since they were elicited only on specific challenge of previously sensitized birds; birds challenged with the heterologous, but related, species *E. acervulina* did not respond in this way, and unimmunized birds did so only after receipt of materials prepared from immunized birds. Serum plasma and extracts of circulating leukocytes (>95% lymphocytes) consistently transferred a leukopenic response, and leukocytosis was seen when the recipients were sampled at 7 h. Viable spleen cells also transferred the leukopenic response to challenge in the one experiment carried out.

TABLE 4. Effect of inoculation with oocysts of *E. maxima* on PBL counts in line 6 recipients of spleen cells from immunized donors

Treatment	No. of circulating leukocytes ($10^3/\mu l$)[a]					
	Time (h) after inoculation of oocysts				Decline (0 to 1 h)	Recovery (1 to 7 h)
	0	1	2	7		
Immune spleen cells[b] and challenge[c]	22.5	17.4	22.3	22.5	5.1[d]	5.1[e]
Challenge[c] only	18.6	17.7	17.9	17.8	0.9	0.1

[a] Values are means; there were four birds per group. Analyses of variance, trends over time, were examined by evaluating differences corresponding to the declining (0- to 1-h), and the recovery (1- to 7-h) phases. Significance tests were based on variations between birds (6 df). The significances were $P > 0.05$ (not significant) at 1 h after inoculation; $0.01 < P < 0.05$ at 0, 2, and 7 h after inoculation, $0.001 < P < 0.01$ at decline; and $P < 0.001$ at recovery. The standard errors were 0.83, 0.66, 0.93, 1.33 for 0, 1, 2, and 7 h, respectively, after inoculation; 0.61 for decline; and 1.11 for recovery.

[b] 6×10^8 cells were given intravenously 7 days before challenge.

[c] 8×10^5 oocysts were given orally.

[d] $P < 0.001$.

[e] $0.001 < P < 0.01$.

The nature of the stimulus(i) present in the transferred materials could not be determined in these experiments: serum could contain both antibodies and other products of leukocytes, either secreted or released as a result of damage during clotting; plasma would contain antibodies, other materials secreted by leukocytes, but probably not materials released from damaged cells, which should be the major constituents of the leukocyte (>95% lymphocyte) extracts; viable spleen cells (lymphocytes, 70%; large mononuclear cells, 27%; heterophils, 3%) would be capable of secreting both antibodies and lymphokines. It seems likely that a complex of factors is involved, and the results of previous work have indicated a role for both antibodies (11) and lymphocyte products such as dialyzable transfer factor (4–6) in coccidial infections.

An immune, and similarly serum-transferable, accumulation of neutrophils in intestinal villi and their emigration into the lumen of the gut have been described in pigs immunized with bovine serum albumin and challenged enterally with this antigen (1). The circulatory changes in leukocytes described here may represent a preliminary stage, i.e., depletion of the circulating pool, of a similar phenomenon. An increased accumulation in coccidia-infected gut of intravenously injected, radioisotope-labeled lymphoblasts, derived from the blood circulation, has already been shown (17).

Although the response described here is immunologically mediated and may be a means for promoting contact between host effector cells and the parasite, it is not necessarily involved in protection. That T-lymphocytes are the cells primarily involved is suggestive of a functional protective role, since a deficiency of these cells causes rats (13, 15, 18) and mice (3, 8) to be unable to resist reinfection with their respective *Eimeria* spp. and a late secondary-type response in PBLs is not induced by challenge of previously infected athymic rats (15). Also correlating with protective immunity, and demonstrated here, was the species specificity of the response. This was surprising in view of the antigenic cross-reactions found in the *Eimeria* spp. but may reflect differences between species in the expression of antigens (20). Contrary indications for the involvement of the leukocyte response in protective immunity are provided by the results of preliminary (unpublished) experiments. These indicated that, although birds can be sensitized by the intestinal application of soluble antigen to give the characteristic leukocyte response to challenge with oocysts, they are fully susceptible to infection. It is of course possible that the antigen used for this sensitization was inappropriate.

Further work is needed to determine the nature of the stimulus for mobilization and the origin, destination, and function of the heterophils and lymphocytes involved.

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