Inhibition of the development of *Eimeria tenella* in cultured bovine kidney cells by a soluble factor produced by peripheral blood lymphocytes from immune chickens

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SUMMARY

The intracellular development of *Eimeria tenella* sporozoites in *in vitro* cultured Madin–Darby Bovine Kidney (MDBK) cells was inhibited when parasite-infected MDBK cells were incubated with peripheral blood lymphocytes (PBL) from infected chickens. The inhibition mediated by PBL was quantified by [^{3}H]uracil uptake and increased during the course of a series of oral infections of chickens with *E. tenella*. This was mirrored by the development of immunity in these birds, as assessed by counting the oocyst output following each re-infection. Similar levels of inhibition were observed using PBL from 3 inbred lines of chickens which differ in their relative susceptibility to infection with *E. tenella*, indicating that the genetic background of the host does not influence the production of this inhibitory activity. The inhibition could be transferred to freshly infected MDBK cells using supernatants prepared from parasite-infected monolayers incubated for 48 h with PBL from immune chickens. However, there was no inhibition using either supernatants from infected MDBK cells incubated with PBL from uninfected chickens, or supernatants from uninfected MDBK cells incubated with PBL from immune chickens. Experiments using Transwell plates showed that direct contact of PBL from immune birds with infected MDBK monolayers was not required to produce supernatants with inhibitory activity. Thus production of soluble inhibitory factor(s) by PBL from immune chickens can be specifically induced by soluble antigens present in the culture media of parasite-infected MDBK cells. These factors inhibit the intracellular development of sporozoites in *in vitro* culture.

Key words: *Eimeria tenella*, immunity, inhibition, development, *in vitro*, soluble factor.

INTRODUCTION

Infection with parasites of the genus *Eimeria* induces immune responses which lead to protection against re-infection with the same species of parasite. Although immunity can operate against all of the developmental stages, the primary target in the immune animal is the sporozoite. For *Eimeria tenella*, sporozoite invasion of the caecum is reduced in immune chickens compared to non-immune controls (Augustine & Danforth, 1986). Those sporozoites which do invade cells, fail to develop into schizonts and remain within the lamina propria (Vervelde, Vermeulen & Jeurissen, 1995) or within transporting lymphocytes in the crypts (Rose, Lawn & Millard, 1984). The range of immune responses induced by *Eimeria* spp. is wide and although the mechanisms that mediate protection are not clearly defined, it is known that acquired immunity is T-cell dependent (for reviews see Wakelin & Rose, 1990; Lillehoj & Trout, 1994; Ovington, Alleva & Kerr, 1995) and that antibodies make only a small contribution to overall immunity.

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The genetic background of the host influences resistance to infection and for *E. tenella* significant differences in oocyst outputs can be measured between inbred lines of chickens (Bumstead & Millard, 1987; 1992; Lillehoj *et al.* 1989*a*).

Recently we have shown significant differences between inbred lines of chickens in the *in vitro* proliferative responses of their peripheral blood lymphocytes (PBL) to *E. tenella* sporozoite antigens. These differences correlate directly to the genetic resistance of the host and to the level of acquired immunity to *E. tenella* (Bumstead *et al.* 1995*a*; Bumstead, Bumstead & Tomley, 1995*b*).

The complexity of *in vivo* immune responses to infection makes it difficult to analyse the contribution of specific responses to overall immunity. This is compounded in chickens since very few immunological reagents and genetically altered host strains are available. *In vitro* cell culture models of infection with *E. tenella* have been used to investigate anti-parasite activities associated with T-cells or their soluble products. Most of this work has used primary chick kidney cells but it has also been noted that growth of parasites in bovine kidney cells is inhibited by soluble factors produced by immune chicken T-cells stimulated *in vitro* with sporozoite antigens

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(Lillehoj *et al.* 1989*b*). In this paper, we have studied the inhibition of parasite development in Madin–Darby Bovine Kidney (MDBK) cells by PBL taken from infected and uninfected chickens to determine whether the inhibitory activity is specific, whether host genetic background or the level of anti-parasite immunity influences the level of inhibitory activity and finally whether direct contact between PBL and parasite-infected MDBK cells is required for either the induction or the function of the inhibitory activity.

MATERIALS AND METHODS

Parasite and chickens

The Wisconsin strain of *E. tenella* (McDougald & Jeffers, 1976) was maintained by serial passage through 8-week-old Light Sussex chickens. Oocysts were propagated, isolated and sporulated as described (Long *et al.* 1976) and sporozoites purified by DE-52 anion-exchange chromatography (Schmatz, Crane & Murray, 1984). For experimental infections, 2 White Leghorn lines (N and 6) and a Brown Leghorn line (BrL) were used. All chickens were housed and reared under coccidia-free conditions.

Experimental design

Trials to standardize conditions for a [^{3}H]uracil uptake assay (Schmatz, Crane & Murray, 1986) and to determine optimal numbers of PBL to be added to *in vitro* cultures, were carried out using PBL from Light Sussex chickens 15 days after a second infection with *E. tenella* and from uninfected age-matched controls. Previous work (Bumstead *et al.* 1995*a*, *b*) had shown that PBL isolated at this time have good responses to *E. tenella* sporozoite antigens in *in vitro* lymphoproliferative assays. Longitudinal studies to examine the inhibitory effects of PBL on parasite development *in vitro* were carried out using groups of 5, 4-week-old chickens of Lines N, 6 and BrL. Birds were either given 3 successive oral infections with sporulated oocysts of *E. tenella* (first dose 100; second dose 10000, third dose 50000) or housed separately and maintained as uninfected, age-matched controls. The total oocyst output from each bird following each infection was calculated by daily counting of oocysts in diluted faeces collected between 5 and 10 days p.i.

In vitro *culture of parasites*

MDBK cells (ICN Flow) were maintained at 37 °C, 5% CO_2 in Ham's culture medium (Ham's F-12 Nutrient mixture, Gibco–BRL, supplemented with 1% L-glutamine, 10% foetal calf serum, 200 units/ml penicillin, 20 μg/ml streptomycin) and passaged twice-weekly. For infection with *E. tenella*, MDBK cells were suspended in Ham's culture medium at 6×10^5 cells/ml and 100 μl volumes dispensed into the wells of flat-bottomed 96-well plates and incubated for 2 h at 41 °C. To each well, freshly purified *E. tenella* sporozoites suspended in Ham's culture medium (various concentrations in a volume of 100 μl, see Results section), were added and the plates re-incubated for a further 2 h. The wells were carefully washed 3 times to remove extracellular sporozoites and non-adherent MDBK cells, then the plates were re-incubated at 41 °C, 5% CO_2 in fresh Ham's culture medium with 5% foetal calf serum.

Intracellular parasite development was measured by the incorporation of [^{3}H]uracil into nucleic acid (Schmatz *et al.* 1986). Briefly, 1·0 μCi of [^{3}H]uracil was added to each well for a specific labelling period (see Results section), then cells were trypsinized, harvested onto glass fibre filter mats (Dynatron Macromash Harvester, Dynatech Laboratories Ltd), washed and cpm determined using a Beckman scintillation counter. Control wells were set up which contained sporozoites alone or MDBK cells alone. All assays were carried out in triplicate.

Parasite invasion was directly measured in infections which were set up in 24-well plates (Nunc) containing glass cover-slips, using the same cell:sporozoite ratios as in the [^{3}H]uracil uptake assays. Cover-slips were removed, fixed in methanol and acetone, stained with haemotoxylin and eosin, mounted under polyvinyl resin and examined microscopically. The total numbers of intracellular parasites within 10 random fields at $\times 40$ magnification were counted for each cover-slip.

Isolation of PBL

Blood was withdrawn from superficial wing veins and PBL were isolated from each bird by centrifugation (Bumstead *et al.* 1995*a*). PBL were washed 3 times in Hams culture medium and resuspended in Hams ready for use at various concentrations.

Inhibition of parasite development by PBL

MDBK cells were infected with sporozoites as described above. Two h p.i., monolayers were washed to remove extracellular sporozoites and to each well 100 μl of PBL from a single bird were added. Cells were re-incubated for various times, as indicated in the Results section. Incorporation of [^{3}H]uracil was determined for each well as described and the effect of PBL on parasite development was calculated by comparison of incorporation achieved in wells incubated with PBL from infected chickens

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with wells incubated with PBL from uninfected chickens. Results were displayed graphically as:

$$1-\left[\frac{\text{Mean cpm of infected MDBK incubated with PBL from infected chickens}}{\text{Mean cpm of infected MDBK incubated with PBL from uninfected chickens}}\right]\times 100.$$

The log-transformed data were used in a 1-way analysis of variance to compare responses between infected and uninfected birds for each line of birds. Control wells were set up containing either sporozoites alone, MDBK alone, PBL alone or sporozoites with PBL.

Inhibition of parasite development by soluble products of PBL

Two h p.i. of MDBK cells, 100 μl volumes of PBL, taken from either infected or uninfected chickens were added to wells and the plates re-incubated for 48 h at 41 °C at which time supernatants (conditioned media) were removed, centrifuged at 5000 ***g*** for 10 min and stored at −20 °C until use.

Infected MDBK cells were also set up using the same relative MDBK and sporozoite concentrations within 24-well companion plates (Transwell, Falcon, Becton-Dickinson). Two h p.i., PBL from either infected or uninfected chickens were added to inserts (pore size 0·45 μm) that were suspended in the medium above the MDBK monolayer so that there was no direct contact between the 2 sets of cells. Supernatants (conditioned media) were removed at 48 h p.i., centrifuged at 5000 ***g*** for 10 min and stored at −20 °C until use.

Fresh monolayers of MDBK cells were infected with sporozoites as described above and at 2 h p.i., normal Ham's medium was supplemented with 50 % (v/v) conditioned medium. Incorporation of [^{3}H]-uracil was determined for each well as described and the effect of conditioned media derived from co-cultures of infected MDBK and PBL from infected chickens was calculated by comparison with wells incubated in conditioned media derived from co-cultures of infected MDBK and PBL from uninfected chickens. Results were displayed graphically as:

$$1-\left[\frac{\text{Mean cpm when incubated with CM from infected MDBK and PBL from infected chickens}}{\text{Mean cpm when incubated with CM from infected MDBK and PBL from uninfected chickens}}\right]\times 100.$$

Supernatants from sporozoites alone, PBL alone, uninfected MDBK and uninfected MDBK co-cultured with PBL from infected chickens were used as additional controls.

RESULTS

Lines N, 6 and BrL differ in respect to their oocyst outputs following infection

These lines of chicken differ from each other in their relative susceptibilities to *E. tenella* as assessed by the numbers of oocysts excreted in the faeces following primary or secondary infections (Bumstead & Millard, 1992; Bumstead *et al*. 1995*a*). This was confirmed in the current experiments (Table 1) with line N birds producing significantly fewer oocysts than the other 2 lines after either 1 or 2 infections with *E. tenella*. After the third infection, none of the birds excreted more than a few oocysts.

Titration of [^{3}*H*]*uracil incorporation by* E. tenella *within MDBK*

Conditions for the assay were determined in 2 trials where MDBK were infected with a range of concentrations of sporozoites and pulse-labelled with [^{3}H]uracil over different time-periods. The mean cpms for each treatment are displayed in Table 2 (Trials 1 and 2). In Trial 1 incorporation of [^{3}H]uracil increased for all time-periods as the concentration of infecting sporozoites was increased from 0·25 to 1×10^6/ml. Increasing the concentration to 2×10^6/ml reduced incorporation, compared to using sporozoites at 1×10^6/ml, and this reduction was attributed to monolayer damage caused by the heavy load of invading parasites. The highest levels of incorporation were measured between 24 and 44 h p.i. which is the time at which 1st-generation schizonts are maturing within the infected cells. In the second trial, using a single labelling period of 24–44 h, incorporation again increased with sporozoite concentration and, in this trial, there were no apparent deleterious effects to the monolayer using sporozoites up to 2×10^6/ml. There was no significant incorporation of [^{3}H]uracil by either sporozoites alone or MDBK alone in either trial. Extensive washing of infected monolayers does not remove all extracellular sporozoites. To determine if inhibitory effects of PBL are directed against intracellular development, rather than invasion, it is important to know whether these extracellular sporozoites account for a significant amount of invasion after PBL are added. In Trial 2, intracellular parasites were counted microscopically at 2 and 24 h p.i. with either 1×10^6 or $0{\cdot}25\times10^6$/ml sporozoites. At both of these multiplicities there was a small increase in the numbers of intracellular parasites at 24 h compared to those at 2 h. Overall this indicates that 88 % of total sporozoite invasion occurs by 2 h post-infection. Thus inhibitory effects of PBL added at 2 h p.i. are mainly attributable to effects on intracellular development rather than parasite invasion. Immunofluorescence studies in our lab-

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Table 1. Oocyst excretion in 3 lines of chickens following 3 oral infections with sporulated oocysts of *Eimeria tenella*

	Oocysts in faeces ($\times 10^{-6}$)* following dose of		
Group	100 (day 0)	10000 (day 21)	50000 (day 36)
Line 6	148·2 ± 41·3	52·3 ± 26·3	0·12 ± 0·001
Line N	46·5 ± 23·7	8·2 ± 6·3	0·01 ± 0·005
Line BrL	151·5 ± 58·2	25·6 ± 12·8	1·05 ± 0·04

* Mean numbers and standard errors for 5 birds/group.

Table 2. Uptake of [^{3}H]uracil following labelling of cultured MDBK cells infected with sporozoites of *Eimeria tenella* and enumeration of intracellular parasites at 2 and 24 h post-infection

Trial 1 Sporozoites ($\times 10^6$/ml)	Counts/min* after pulse time of		
	0–24 h	24–44 h	44–70 h
2	7112 ± 536	12030 ± 1736	3326 ± 1036
1	9054 ± 1023	15174 ± 1976	9892 ± 772
0·5	4923 ± 763	6034 ± 1055	4377 ± 403
0·25	3576 ± 264	4125 ± 732	4476 ± 377
MDBK only	213 ± 43	239 ± 56	226 ± 41
Sporozoites only (2×10^6/ml)	199 ± 41	175 ± 48	151 ± 57
Trial 2 Sporozoites ($\times 10^6$/ml)	Counts/min* 24–44 h	Intracellular parasites† at 2 h	24 h
2	15063 ± 1505	N.D.	N.D.
1	11154 ± 973	898 ± 59	1021 ± 45
0·5	5921 ± 624	N.D.	N.D.
0·25	3864 ± 504	266 ± 28	303 ± 31
MDBK only	305 ± 79	N.D.	N.D.
Sporozoites only (2×10^6/ml)	214 ± 56	N.D.	N.D.

* Mean numbers and standard errors for [^{3}H]uracil uptake in 3 replicate wells.
† Mean numbers and standard errors for the counts from 10 random microscopic fields at ×40 magnification.
N.D., Not determined.

Table 3. Percentage inhibition of the uptake of [^{3}H]uracil by cultured MDBK cells infected with sporozoites of *Eimeria tenella* by the addition of PBL from either immune or non-immune chickens

Trial 3	Percentage inhibition* of [^{3}H]uracil uptake by the addition of	
Number of cells added ($\times 10^7$/ml)	Immune PBL	Non-immune PBL
2	72	35
1	68	14
0·5	30	12

* Results expressed as the percentage inhibition of [^{3}H]uracil uptake in sporozoite-infected MDBK cultures to which PBL were added compared with parallel cultures incubated without PBL.

oratory have also shown that using this *in vitro* infection system the majority of sporozoites invade the MDBK within a few minutes of being added to the cells (Tomley *et al*. 1996). For all the remaining assays a sporozoite concentration of 1×10^6/ml with a labelling period between 24 and 44 h was used.

Inhibition of parasite development by immune PBL

A third series of assays (Table 3), using PBL from immune and non-immune Light Sussex chickens, was used to establish the optimal number of PBL to add to each well. Inhibition of [^{3}H]uracil uptake by parasite-infected MDBK increased with increasing numbers of PBL from immune chickens (Table 3) with 30% inhibition at $0{\cdot}5 \times 10^7$/ml and up to 72% at 2×10^7/ml. However, at the highest concentration a significant amount of inhibition (35%) also

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Table 4. Uptake of [^{3}H]uracil by cultured MDBK cells infected with sporozoites of *Eimeria tenella* after the addition of PBL from either infected (I) or non-infected (NI) chickens

Day post-primary infection		Counts/min*		
Experiment 1		Line N	Line 6	Line BrL
0	I	5198 ± 543	4863 ± 876	5022 ± 567
	NI	5092 ± 987	4911 ± 745	5072 ± 781
18	I	7309 ± 1067	6892 ± 406	6259 ± 578
	NI	7166 ± 1122	7309 ± 682	6589 ± 765
20	I	4825 ± 568	3313 ± 543	3621 ± 733
	NI	4583 ± 812	4872 ± 763	5029 ± 234
32	I	3617 ± 345	2031 ± 249	2578 ± 443
	NI	9521 ± 852	8123 ± 478	8593 ± 780
36	I	3847 ± 456	3015 ± 118	4659 ± 641
	NI	7125 ± 349	6854 ± 786	7059 ± 1023
Experiment 2				
0	I	5806 ± 688	6015 ± 553	7132 ± 689
	NI	5778 ± 956	6024 ± 586	7146 ± 991
13	I	6538 ± 554	5399 ± 789	5788 ± 783
	NI	7265 ± 762	7104 ± 882	7421 ± 878
28	I	3935 ± 389	4783 ± 712	3901 ± 829
	NI	6054 ± 976	6932 ± 873	6096 ± 803
36	I	5905 ± 877	5955 ± 567	5892 ± 516
	NI	8437 ± 1132	8158 ± 998	7963 ± 912
50	I	3628 ± 677	3265 ± 569	4821 ± 391
	NI	6478 ± 723	7421 ± 984	7305 ± 987

occurred when infected MDBK were incubated with PBL from uninfected chickens. The cause of this non-specific inhibition was not investigated further, and for all future assays a PBL concentration of 1×10^7/ml was used.

For the 2 longitudinal studies (Exps 1 and 2) PBL were isolated, at various times after a first, second or third infection with *E. tenella* and from uninfected controls. PBL were added to infected MDBK and [^{3}H]uracil uptake measured as described (see Table 4). For ease of comparison between the different time-points during the course of these experiments, results are also displayed as the mean percentage inhibition of [^{3}H]uracil uptake in cultures containing PBL from infected chickens, compared to cultures containing PBL from uninfected chickens (Fig. 1). In Exp. 1 (Fig. 1A), there was no inhibition of [^{3}H]uracil uptake by PBL taken from any of the birds pre-infection or 18 days following primary infection. At 2 days after a second infection uptake was significantly inhibited by PBL from 2 of the lines, and from all 3 of the lines (mean 72% for all the lines) at 14 days. After a third infection, PBL taken 4 days later from all lines inhibited uptake but to a lesser extent (mean 45% for all the lines). The only statistically significant difference between the 3 lines was in the response at 2 days after the second infection when PBL from Line N birds did not inhibit uptake. In Exp. 2 (Fig. 1B) samples were taken at later time-points after both second and third infections. In this experiment, some inhibitory activity was detected 13 days after the primary infection and again at both 15 and 23 days after the second infection and at 14 days after the third infection. There were no significant differences in inhibition by PBL between the lines at any of the sampling times. The level of inhibition increased following each infection, although overall the amount of inhibition was less than in Exp. 1. For both experiments control wells set up with sporozoites alone, MDBK alone or PBL alone did not incorporate [^{3}H]uracil. However, wells set up with PBL incubated with sporozoites incorporated small amounts of isotope label, possibly indicating that sporozoites can invade PBL and are capable of some intracellular metabolism within these cells.

Inhibition is mediated by a soluble factor and cell–cell contact is not needed for its production

PBL taken from birds during the course of Exp. 2 were also used to investigate whether inhibition of [^{3}H]uracil uptake by PBL requires that the PBL be in direct contact with infected MDBK cells or whether soluble factor(s) are involved. Supernatants were removed from a series of infected MDBK monolayers to which PBL from infected or non-infected chickens were added for 48 h and stored at −20 °C. The ability of these conditioned media to inhibit the development of parasites within freshly infected MDBK was assayed by [^{3}H]uracil uptake. The cpms, standard deviations and day to day

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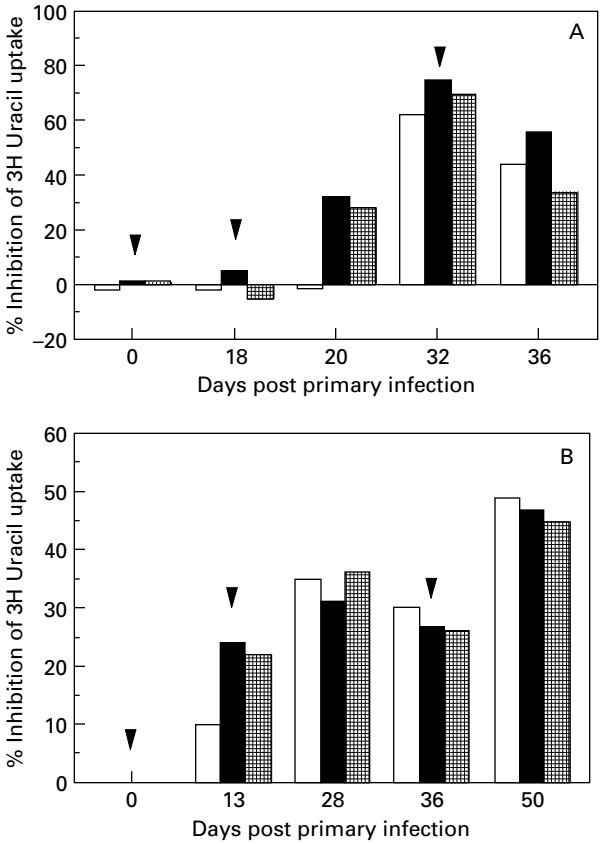

Fig. 1. Inhibition of the uptake of [^{3}H]uracil by cultured MDBK cells infected with sporozoites of *Eimeria tenella* by the addition of PBL from line N (□), line 6 (■) and line BrL (▦) chickens. Birds (5/group) were given 3 successive oral infections with 100, 10000 and 50000 sporulated oocysts of *E. tenella* (indicated by solid arrowheads), or were mock infected. PBL were removed from all birds at the times indicated and the inhibition of [^{3}H]uracil uptake mediated by PBL from infected birds determined by comparison to parallel cultures incubated with PBL from mock infected birds (3 replicates/bird). (A)(Experiment 1: birds were infected on days 0, 18 and 32; PBL were removed on days 0, 18, 20, 32 and 36. (B) Experiment 2: birds were infected on days 0, 13 and 36; PBL were removed on days 13, 28, 36, and 50. Results are expressed as percentage inhibitions, calculated for each line of chickens from the mean cpm of sporozoite-infected MDBK incubated with PBL from infected chickens and the mean cpm of infected MDBK incubated with PBL from mock infected chickens.

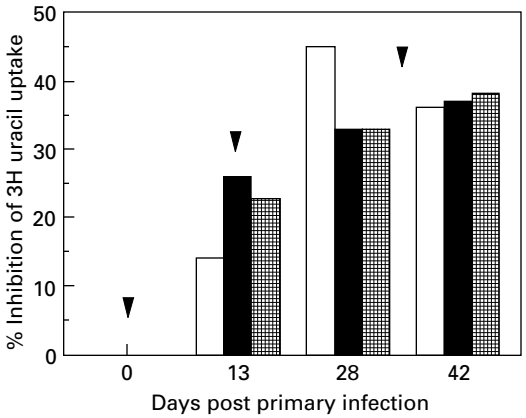

Fig. 2. Inhibition of the uptake of [^{3}H]uracil by cultured MDBK cells infected with sporozoites of *Eimeria tenella* by the addition of supernatants (conditioned media, CM) from direct co-cultures of infected MDBK cells and PBL from line N (□), line 6 (■) and line BrL (▦) chickens. Samples are from Exp. 2 in which birds were infected on days 0, 13 and 36 (indicated by solid arrowheads) and PBL were removed on days 13, 28, 36 and 42. Results are expressed as percentage inhibitions, calculated for each line of chickens from the mean cpm of sporozoite-infected MDBK incubated with CM from co-cultures of infected MDBK with PBL from infected chickens, and the mean cpm of infected MDBK incubated with CM from co-cultures of infected MDBK with PBL from mock infected chickens.

variation in all subsequent assays were of similar magnitudes to those obtained using whole PBL and the raw data are not presented. The results are expressed as the mean percentage inhibition of [^{3}H]uracil uptake by supernatants transferred from cultures incubated with PBL from infected birds, compared to supernatants transferred from cultures incubated with PBL from uninfected birds (5 birds/group, 3 replicate treatments/assay). Fig. 2 shows that supernatants transferred from infected MDBK cells co-cultured with PBL from birds after primary, second or third infections were all capable of inhibiting [^{3}H]uracil uptake by parasites developing in freshly infected MDBK cells. There were no significant differences, between the 3 lines of chickens and inhibition induced by the transferred supernatants was broadly similar to that achieved by direct contact of PBL with infected MDBK cells within the same experiment (compare Fig. 2 with Fig. 1B). This indicates that the inhibition is caused by soluble factor(s) and that direct interaction of PBL with infected MDBK cells is not required.

To determine whether direct contact between PBL and infected MDBK was necessary to induce the production of soluble inhibitory factor(s), a second parallel series of supernatants was collected from infected MDBK monolayers set up within Transwell companion plates. In these plates, the PBL from either infected or non-infected chickens were contained within inserts suspended above the infected monolayer so that the cells shared the same medium but there was no direct cell to cell contact. Fig. 3 shows that these supernatants, produced in parallel to the supernatants where direct contact between PBL and MDBK was permitted (Fig. 2), were also capable of inhibiting [^{3}H]uracil uptake by freshly infected MDBK and to a similar level. Thus, it appears that direct contact between the PBL and the infected MDBK is not required to induce the production of the inhibitory activity.

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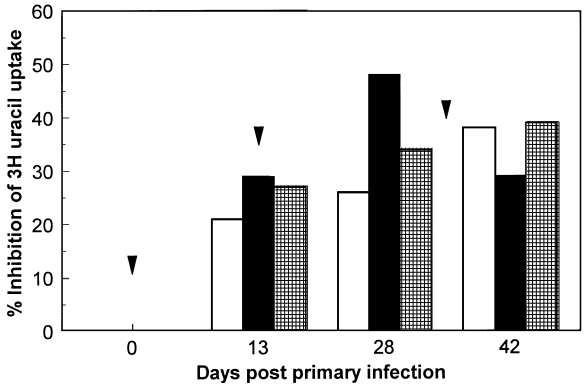

Fig. 3. Inhibition of the uptake of [^{3}H]uracil by cultured MDBK cells infected with sporozoites of *Eimeria tenella* by the addition of supernatants (conditioned media, CM) taken from Transwell co-cultures of infected MDBKL cells and PBL from line N (□), line 6 (■) and line BrL (▦) chickens. Samples are from Exp. 2 in which birds were infected on days 0, 13 and 36 (indicated by solid arrowheads) and PBL were removed on days 13, 28, 36 and 42. Results are expressed as percentage inhibitions, calculated for each line of chickens from the mean cpm of sporozoite-infected MDBK incubated with CM from Transwell co-cultures of infected MDBK with PBL from infected chickens, and the mean cpm of infected MDBK incubated with CM from Transwell co-cultures of infected MDBK with PBL from mock-infected chickens.

For both assays control wells containing sporozoites alone or MDBK alone did not incorporate [^{3}H]uracil. Supernatants from sporozoites alone, PBL alone, uninfected MDBK and, most importantly, uninfected MDBK co-cultured with PBL from immune birds did not inhibit [^{3}H]uracil uptake by freshly infected MDBK cells.

DISCUSSION

The intracellular development of *E. tenella* is readily quantified in MDBK cells by measurement of [^{3}H]uracil incorporation into nucleic acid (Schmatz *et al*. 1986). This assay offers advantages over microscopical methods since it requires less material, is objective and allows rapid processing of many replicates of treatments. However, the assay is not suitable for use in primary chick kidney cells because of high background levels of [^{3}H]uracil incorporation by uninfected cells (results not shown). We used 2 separate experiments to determine the inhibition of parasite development within MDBK cells mediated by PBL which were taken from chickens during a course of oral infections with the homologous parasite. Inhibition of development by PBL was detected in 1 experiment after a single oral infection with *E. tenella* and increased throughout both experiments as immunity to the parasite developed. Immunity was assessed by the decrease in oocyst output following each re-infection. PBL from infected chickens of 3 different inbred lines were all effective in inhibiting parasite development *in vitro* indicating that the inhibition is not influenced by the genetic background of the host even though these lines consistently show significant differences in their resistance to infection with *E. tenella* and in their lymphoproliferative responses to parasite antigens (Bumstead *et al*. 1995*a*, *b*).

In infected MDBK cell cultures, direct counting of intracellular parasites at 2 and 24 h post-infection showed that the majority (88 %) of parasite invasion occurred by 2 h. Thus, by adding PBL at 2 h p.i., inhibition of [^{3}H]uracil uptake measured from 24 to 44 h p.i. can largely be attributed to inhibition of intracellular development rather than prevention of invasion.

Inhibition was mediated by soluble factor(s) produced in the *in vitro* culture by PBL taken from infected, but not from uninfected, chickens. Direct contact with the infected MDBK monolayer was not needed for the production of the inhibitory factor(s) since supernatants from Transwell plates mediated inhibition in freshly infected MDBK cells at a level similar to supernatants from direct co-cultures. However, inhibition is antigen specific because PBL from infected birds incubated, either directly or in Transwell plates, with uninfected MDBK monolayers do not produce conditioned media capable of inhibiting parasite development. The inhibition was not due to specific anti-*E. tenella* antibodies in the PBL suspensions as there was no reactivity of the suspensions on Western blots or ELISA (data not shown).

There have been a number of reports on the inhibition of growth of *Eimeria* spp. in culture using soluble products from immune lymphocytes. The specific effects of interferon-γ on *E. tenella* have been studied in bovine and human cell cultures by treatment with, respectively, bovine or human recombinant interferon-γ (Kogut & Lange, 1989*a*, *b*) and clearly show that interferon-γ has no direct anti-sporozoite activity. Instead, inhibition is mediated *via* the host cell and treatment of the host cells prior to infection is required to induce inhibitory effects. It is unlikely that the inhibitory effects of immune PBL described here are mediated by interferon-γ since pre-treatment of the infected cells was not required for inhibition and the levels of interferon-γ in culture supernatants did not correlate with inhibition (results not shown). Furthermore it is thought highly unlikely that the functional activity of interferon-γ can cross the species barrier (Grossberg, 1987; Lowenthal, York & Digby, 1994). A detailed study of a soluble anti-*E. tenella* inhibitory activity produced by *in vitro* mitogen stimulated immune chicken spleen cells (Kogut & Slajchert, 1992) has concluded that a factor additional to interferon-γ is involved.

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It has previously been shown that concanavalin A-stimulated normal chicken spleen cell supernatants and sporozoite antigen-stimulated immune chicken spleen cell supernatants can inhibit the intracellular development of *E. tenella* in bovine cells (Kogut & Lange, 1988; Lillehoj *et al*. 1989*b*). We have now shown that significant levels of inhibition can be detected when PBL are added directly to infected monolayers, without pre-treatment of the host cells and that the amount of inhibition is directly related to the level of immunity in the chicken, as judged by oocyst excretion. The factor(s) described here appears to be different from transfer factor (Klesius & Giambrone, 1984) which is a non-secreted dialysable extract obtained from lysed immune T-lymphocytes. However, further comparative studies would be of interest as transfer factor has also been reported to transfer immunity in heterologous systems across host species (Klesius, 1982).

It was recently reported that the *in vitro* development of *E. tenella* in chicken cells could be inhibited by direct co-cultivation of infected monolayers with immune spleen cells (Miller, Bowman & Schat, 1994). These authors showed that immune splenocytes are induced rapidly and are not restricted by the MHC and suggested that a cellular mechanism, possibly natural killer cell activity, was responsible for the inhibition. However, the production of soluble inhibitory factors by immune cells was not assayed. In our study inhibition appears to be mediated by a soluble factor produced by immune cells but direct contact between infected monolayers and immune cells is not required for either its induction or activity. It is known that soluble parasite antigen(s) are released into the cell culture medium during the early stages of parasite invasion and development in host cells (Clare *et al*. 1993; Tomley *et al*. 1996). If a similar phenomenon occurs *in vivo* then these proteins represent potential targets for early cellular immune responses and could induce immunity. In the context of our experiments it is tempting to speculate that soluble parasite antigens may be responsible for the induction of the observed inhibitory activity and studies to characterize such antigens are currently in progress. The PBL preparations that we have used are not purified and therefore are likely to contain cells (for example macrophages and B-cells) capable of processing exogenous antigens and presenting them to lymphocytes via the MHC class II pathway. Specific enrichment or depletion of cells from the PBL should provide valuable information on the role of different cell populations in the production of inhibitory factors.

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