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Effect of the parasite enzyme, hypodermin A, on bovine lymphocyte proliferation and interleukin-2 production via the prostaglandin pathway

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SUMMARY

The immune function of cattle infected with a primary infestation of *Hypoderma lineatum* is impaired during the first instar migration of the larvae. Hypodermin A (HA) is an enzyme secreted by the larvae that is implicated in immunosuppression. The response of bovine peripheral blood mononuclear cells (PBMC) to HA was examined in this study. HA blocked their proliferation in response to phytohaemagglutinin (PHA) and its effect was enhanced when cells were preincubated with HA before activation. This suggests that HA affects the lymphocyte commitment to blastogenesis during the early stages of their activation. HA also markly reduced the production of interleukin-2 (IL-2) in PHA-stimulated bovine PBMC cultures. Furthermore, indomethacin, which inhibits prostaglandin (PG) synthesis, blocked the immunosuppressive effect of HA on the PBMC proliferative response. The concentration of PGE_2 in medium of PBMC or PMA-stimulated monocyte cultures was increased by incubation with HA. Thus, the HA appeared to act by reducing IL-2 production via a prostaglandin-dependent pathway.

INTRODUCTION

Bovine hypodermosis is a parasitic disease that causes economic loss by reducing the zootechnical performances of livestock. It has also been demonstrated in recent years that infestations of cattle with *Hypoderma lineatum* are associated with alterations of the host's immune system. This immunomodulation impairs both the inflammatory system[1,2] and the specific immune system.[3,4] Animals suffering from a primary infestation have a reduced antigen-specific or mitogen-induced lymphocyte responsiveness during the early phase of infestation, which results in a significant number of larvae reaching maturity. This suppression of specific and non-specific cellular immune functions elicited by *H. lineatum* takes place when larvae of the first stage are migrating to the backs of the cattle.[5] During its migratory phase, the parasite releases three serine proteases, hypodermins A, B and C. It has been demonstrated that these secreted larval enzymes are involved in the control of larva survival and in escape from the cellular immune system.

Recent *in vitro* and *in vivo* studies have shown that hypodermin A (HA) induced positive or negative changes in lymphocyte proliferative responses, that varied with the mitogen used to stimulate the lymphocyte. The factors or mechanisms by which HA causes immunomodulation are still not clear. This study uses a model of the lymphocyte proliferative system, in which normal bovine peripheral blood mononuclear cells (PBMC) are stimulated with phytohaemagglutinin (PHA). It has been demonstrated that HA inhibits the proliferative response in *in vitro* and *in vivo* studies.[3] We have investigated the mechanisms by which HA inhibits the primary T-cell response. This model could be suitable for demonstrating the events that contribute to immune deregulation during hypodermosis.

This work identifies the step in the stimulation of the PBMC at which HA exerts its maximum modulatory effect. It also traces the secretions of interleukin-2 (IL-2), a molecule that is essential for the lymphocyte proliferation, and the prostaglandin E_2 (PGE_2), a marker of the monocyte activation which participates in the regulation of IL-2 secretion.

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MATERIALS AND METHODS

Animals

The cells used in this study were from clinically healthy Holstein steers obtained from the Institut National de la Recherche Agronomique (INRA, Nouzilly, France) breeding herd. The animals were maintained in an area free of hypodermosis and checked immunologically for hypodermosis.

HA antigen

HA was purified from crude extracts of first instar larvae of *H. lineatum* by diethylaminoethyl (DEAE) ion-exchange chromatography.[6]

Preparation of bovine cells

Buffy coats were obtained from blood collected from the

jugular vein into EDTA tubes, diluted 1:2 with phosphate-buffered saline (PBS), pH 7·2, layered over Ficoll–sodium diatrizoate (Ficoll–Paque 1077; Pharmacia LKB, Uppsala, Sweden), and centrifuged at 700 *g* for 30 min. The mononuclear cells layers were collected from the interface, resuspended in PBS and washed three times. The viability of the cells was over 98%, as determined by trypan blue dye exclusion. PBMC were resuspended in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (subsequently referred to as complete RPMI), containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco). The monocyte population from PBMC (10 ml at 5×10^6 cells/ml) was enriched by allowing the cells to adhere to sterile plastic Petri dishes for 30 min at 37° (5% CO_2/95% air incubator) in complete RPMI containing 10% FBS. Non-adherent cells were removed by washing twice with warm RPMI-1640. Monocytes were removed by gently scraping the Petri dishes, and washed once in RPMI-1640. Monocyte enrichment was assessed by morphology on Giemsa-stained cytocentrifuge smears, which indicated more than 90% of positive cells. Staining for CD5 with monoclonal antibody (mAb) 8C11, and for B cells with ILA30, indicated that lymphocytes were the main cell contaminants, and that they accounted for < 30% of the total cells.

In vitro *proliferation assay*

PBMC (2×10^5 cells/wells) were plated in triplicate in 200 μl of complete RPMI in 96-well flat-bottomed plates (Nunclon Delta; Nunc, Roskilde, Denmark). Cells were cultured with different concentrations of HA and at various times before or after stimulation with 10 μg/ml PHA (Sigma Chemical Co., St Louis, MO), for 72 hr. Indomethacin (50 μM) and dexamethasone (1 μM) (Sigma), were added to the cultures in some experiments. The proliferation induced by IL-2 was neutralized by supplementing cultures with mAb CAC108A (VRMD Inc., Pullmann, WA), which is specific for the bovine IL-2 receptor (IL-2R) α-chain. Cultures were incubated at 37° in a humidified atmosphere of 5% CO_2/95% air. The cells were then pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; Dupont de Nemours) for the final 6 hr of culture, and label incorporation was assessed by liquid scintillation counting.

Assay for bovine IL-2 activity

Supernatants of PHA-activated PBMC were harvested and stored at −20° until titration. IL-2 activity was measured essentially as reported elsewhere.[7] Serial twofold dilutions of the test supernatants were made in tissue culture medium. The dilutions were placed in 96-well flat-bottomed plates in triplicate, containing 5×10^4 freshly washed 6-day-old concanavalin A (Con A) blasts as responding cells, and incubated for 18 hr. The cells were then pulsed as described above. Means of triplicate values were calculated for each dilution. Six-day blasts proliferated in a dose-dependent manner with IL-2, because addition of 1 μg/ml bovine anti-IL-2R mAb almost completely inhibited [^{3}H]TdR incorporation into these cells in the presence of supernatants from cultures stimulated with Con A.

Assessment of PGE_2 production

10^6 monocytes were cultured in 1 ml complete RPMI supplemented with 10% FBS and 50 ng pyruvate myristate acetate (PMA; Sigma) per well in 24-well tissue culture plates. The PGE_2 concentration in the culture supernatants from PMA-stimulated and non-stimulated monocytes, and PHA-stimulated and non-stimulated lymphocytes, was assayed. Supernatants were collected at various times, centrifuged and frozen at −20°. The amount of PGE_2 in monocyte or lymphocyte culture supernatants was quantified using an enzyme immunoassay (EIA) kit (Boehringer-Mannheim France, Meylan, France). The PGE_2 concentration of the samples was calculated from a standard PGE_2 curve.

Statistical analysis

The [^{3}H]TdR uptake by triplicate cultures was determined, and differences between treatments of the cells were tested either for significance within animals by an analysis of variance, or by Student's *t*-test for the means of data from different animals. $P < 0.05$ was regarded as significant between treatments.

RESULTS

Time-dependent inhibition of mitogen-induced proliferation of bovine PBMC by HA

Preliminary trials showed that the lowest concentration of HA that had a significant effect on the PHA-induced responses was 15 μg/ml. This concentration of HA was used in most subsequent experiments. HA was added to bovine PBMC at five times, 2 days or 1 day before mitogen activation, the same time, or 1 day or 2 days after activation with 10 μg/ml PHA, to appreciate the effects of HA on different states of lymphocyte activation. Three separate experiments were carried out on five animals, and a representative result is shown in Fig. 1. We show the percentage of the control PHA-proliferation responses for each culture. HA added to the PBMC 24 hr or 48 hr before stimulation with PHA inhibited the lymphocyte proliferative responses most effectively (42% of the control

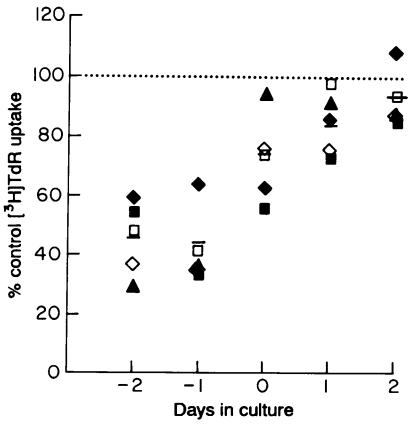

Figure 1. Effects of adding HA before or after PBMC stimulation with PHA. PHA (10 μg/ml) was added day 0; HA was added on days −2, −1, 0, +1 and +2. Three days after PHA stimulus, cultures were pulsed with 1 μCi [^{3}H]TdR for the last 6 hr; the percentage of control refers to the lower PBMC proliferation in the presence of HA than in PBMC without HA from five animals. Results are for one representative experiment out of four.

Table 1. Effect of HA on IL-2 production by PBMC

Supernatant tested	HA	Time of treatment	[^{3}H]TdR incorporation (c.p.m. × 10^{-3}) into 6-day blasts induced by 24-hr or 72-hr supernatants from PBMC cultures				
			1:2	1:4	1:8	1:16	1:32
PHA	–	24 hr	79·7 ± 12	65·8 ± 7·9	52·2 ± 8·3	46·4 ± 6·1	37·8 ± 3·0
	+	24 hr	45·3 ± 2·5*	38·6 ± 6·2*	30·3 ± 4·9*	27·9 ± 1·6*	31·8 ± 1·7
	–	72 hr	93·3 ± 7·1	86·9 ± 0·8	66·8 ± 4·3	43·0 ± 7·8	36·6 ± 5·9
	+						
72*hr*47.71.2 * 48.52.3 * 38.58.1 * 31.11.531.03.4							
PHA ± dexamethasone	–	24 hr	33·3 ± 3·7	21·2 ± 5·5	17·0 ± 4·4	14·7 ± 3·7	11·6 ± 0·7
PHA ± indomethacin	–	24 hr	94·8 ± 15·5	90·9 ± 16·6	86·4 ± 1·8	83·5 ± 17·8	57·3 ± 8·6

The samples tested were 24-hr or 72-hr supernatants of PBMC cultures (2×10^6 PBMC/ml) stimulated with PHA (10 μg/ml) with (+) or without (–) HA (15 μg/ml). The 6-day blast cultures were incubated with these solutions for 18 hr. The cells were pulsed with 1 μCi [^{3}H]TdR for the last 6 hr.
* $P < 0{\cdot}05$, difference with respect to the value obtained without HA.

response after 24 hr and 45·4% after 48 hr). Cells incubated with PHA and HA simultaneously showed 72·5% of the control response. This difference in the PHA-induced response between preincubation with HA and PHA added at the same time as HA was significant for each of the five animals tested ($P < 0{\cdot}05$). Furthermore, HA added to the culture 24 hr or 48 hr after stimulation with PHA produced a non-significant inhibition (means: 84·5% of the control response for 24 hr and 92·7% for 48 hr).

HA inhibited the proliferative response more efficiently when it was added before the mitogen than on the same day. HA did not significantly inhibit lymphocyte proliferation when added after the cell activation.

IL-2 production by PBMC stimulated with PHA in the presence of HA

The production of IL-2 by PHA-stimulated mononuclear cells, with or without HA, added 24 hr before cell stimulation was analysed. The IL-2-like activity in samples of supernatants collected over the 3 days of culture was tested. As illustrated in Table 1, supernatants from PBMC stimulated with PHA, in the presence of HA for 24 hr or 72 hr, contained a significantly less IL-2-like activity than did those from cultures without HA. The difference was more marked for supernatants from 72-hr cultures, because of the higher IL-2 activity in the cultures stimulated with PHA for 72 hr. Control cultures contained dexamethasone, which is known to inhibit IL-2 production, and indomethacin, which causes an increase in IL-2 activity. These controls were needed to test the sensitivity of the IL-2 bioassay.

Bovine anti-IL-2R mAb was added to PBMC stimulated with PHA to analyse the net production of IL-2 by blocking the consumption of newly produced IL-2 by the activated cells (Table 2). The inhibition of IL-2-like activity by HA in the presence of anti-IL-2R was the same with or without anti-IL-2R mAb.

Effect of indomethacin on reduced proliferation by PBMC stimulated with PHA in the presence of HA

The relationship between the depressed PHA responses and IL-2 production of mononuclear cells incubated with HA, and the influence of the prostaglandins, was evaluated using the irreversible inhibitor of cyclo-oxygenase, indomethacin. The addition of indomethacin at 50 μM increased the basal proliferative response of PHA-stimulated PBMC from 147 ± 31·2 to 192 ± 29 × 10^{-3} c.p.m. Cells stimulated with PHA plus HA incorporated over twice as much [^{3}H]TdR with indomethacin (202 ± 35 × 10^{-3} c.p.m.) than without it

Table 2. Effect of HA on the IL-2 activity of supernatants from cells grown in the presence of anti-IL-2R

Time of treatment	HA	[^{3}H] TdR incorporation (c.p.m. x 10^{-3}) into 6-day blasts induced by 72 hr supernatants of lymphoproliferation cultures				
		1:2	1:4	1:8	1:16	1:32
24 hr	–	97·8 ± 12·0	81·5 ± 6·9	66·7 ± 2·7	57·9 ± 7·5	45·5 ± 4·2
	+	40·9 ± 7·0*	33·2 ± 5·0*	39·9 ± 1·7*	32·1 ± 2·8*	29·5 ± 1·9*
72 hr	–	92·9 ± 8·8	88·8 ± 4·0	62·4 ± 1·3	48·1 ± 0·3	40·5 ± 5·5
	+	44·4 ± 4·5*	30·2 ± 6·1*	29·9 ± 2·5*	28·1 ± 5·2*	26·5 ± 1·9*

Anti-IL-2R (1 μg/ml) was added to the cultures to block the lymphocyte proliferation with (+) or without (–) HA (15 μg/ml).
* $P < 0{\cdot}05$, difference with respect to the value obtained without HA.

Table 3. PGE_2 production by bovine mononuclear cells from peripheral blood incubated with HA

Time of supernatant collection	PGE_2 conc. (ng/ml)		
	Control	PHA	PHA + HA
0 hr	3·5 ± 0·13		
24 hr	3·6 ± 0·14	5·7 ± 0·14	6·2 ± 0·12
48 hr	4·0 ± 0·12	7·2 ± 0·13	8·5 ± 0·08*
72 hr	4·5 ± 0·15	9·0 ± 0·34	13·5 ± 0·77*

PBMC from naive calves were incubated with HA (15 μg/ml) and stimulated with PHA (10 μg/ml). PGE_2 levels in the culture supernatants were determined by EIA. Experiments were performed in duplicate ($n = 6$).

* $P < 0·05$ compared with the PHA-stimulated control; Student's *t*-test.

Table 4. PGE_2 production by bovine monocytes/macrophages from peripheral blood incubated with HA

Time of supernatant collection	PGE_2 conc. (ng/ml)		
	Control	PMA	PMA + HA
0 hr	1·2 ± 0·2		
3 hr	1·8 ± 0·1	5·7 ± 0·2	6·0 ± 0·3
6 hr	6·0 ± 0·6	15·2 ± 0·2	30·0 ± 2·2*
24 hr	9·2 ± 0·3	55·0 ± 3·2	75·0 ± 2·2*

Monocytes/macrophages from naive calves were incubated with HA (15 μg/ml) and stimulated with PMA (50 ng/ml). PGE_2 in the culture supernatant was determined by EIA. Experiments were performed in duplicate ($n = 6$).

* $P < 0·05$ compared with the PMA-stimulated control; Student's *t*-test.

($78·3 \pm 21·7 \times 10^{-3}$ c.p.m.). Indomethacin totally restored the proliferative response of cultures given 7·5 μg/ml or 15 μg/ml HA. The responses of cells incubated with HA and the lipooxygenase inhibitor, nordihydroguaiaretic acid (3 μM; NDGA), was also examined (data not shown). This inhibitor never reversed the blockade caused by HA.

Induction of PGE_2 released by HA

As the blockade of the proliferative responses by HA was removed by indomethacin, we sought direct evidence that HA modulated PGE_2 production. We also estimated the amount of PGE_2 contained in the culture medium. The kinetics of PGE_2 production were monitored during a 72-hr culture to study the role of this molecule in inhibiting proliferation. Resting resident PBMC released very low basal levels of PGE_2 *in vitro* (Table 3). In contrast, the concentration of PGE_2 in the medium of mononuclear cell cultures stimulated with PHA increased during the first 3 days of culture, from 5·7 ± 0·14 ng/ml at 24 hr to 9·0 ± 0·34 ng/ml after 72 hr of culture. Cells grown in medium containing both PHA and HA produced greater amounts of PGE_2 than did cells stimulated with PHA without HA, and this was apparent as early as 24 hr. This difference in PGE_2 production with or without HA increased with time in culture, was significantly greater after 48 hr ($P < 0·05$), and was over one and an half times greater with HA at 72 hr of culture (9·0 ± 0·34 ng/ml with PHA and 13·5 ± 0·77 ng/ml with PHA plus HA).

We then focused our investigation on the monocytes, as they are the main cells in PBMC cultures that produce PGE_2. Monocyte-enriched cultures stimulated with PMA showed a markely increased PGE_2 production during the first 24 hr of cultures, increasing from 5·7 ± 0·2 ng/ml at 3 hr of activation to 55 ± 3·2 ng/ml at 24 hr (Table 4). Cells incubated in PMA plus HA produced even more PGE_2 compared to cells stimulated with PMA alone. The concentration of PGE_2 in cells stimulated for 6 hr in PMA alone was 15·2 ± 0·2 ng/ml, while those stimulated in PMA plus HA produced 30 ± 2·2 ng/ml; HA therefore doubled PGE_2 production. HA also significantly increased PGE_2 production ($P < 0·05$) by non-activated monocytes (data not shown). Monocytes incubated for 1 hr in the medium containing 15 μg/ml HA released four times as much PGE_2 than those grown without HA. This suggests that the blockade of the proliferative response to PHA by HA may be due to an early overproduction of PGE_2 by monocytes.

DISCUSSION

We have demonstrated previously that PBMC from uninfected cattle do not proliferate *in vitro* when grown in PHA plus HA.[3] The present report shows that this blockade involves early stages of bovine lymphocyte activation and is associated with a reduction in the IL-2 activity in the culture supernatant by a prostaglandin-dependent mechanism.

The first stage in our attempt to describe the modulation of the defence mechanisms of young cattle during a primary infestation by *H. lineatum* was to analyse the time-dependent response of bovine PBMC *in vitro*. HA blocked the PHA-induced PBMC response more when it was placed in the medium 24 or 48 hr before the mitogen PHA than when it was added at the same time. The reduced response to PHA was not due to increased cell death, as HA appeared to have no effect on cell viability. The HA may interfere with effective lymphocyte triggering or lymphocyte commitment to blastogenesis during the early stages of activation. PHA-mediated activation also appears to depend on the binding of PHA to isolated T-cell receptor (TCR) peptides,[8] and on the expression of the CD2 surface antigen.[9] Therefore, the HA may modulate the expression of these T-cell surface antigen molecules during the early steps of activation.

This early inhibition by parasites or certain parasite secretions has already been described. Lymphocyte proliferation was only inhibited when factors produced by *Schistosoma mansoni* were added at the beginning of the culture.[10] Proliferation was also inhibited when PBMC were incubated with blood forms of *Trypanozoma cruzi* before activation.[11,12] However, *T. cruzi* still significantly inhibited the human lymphocyte response when it was added 48 hr after cell stimulation,[12] whereas HA did not at this time of stimulation. The fact that HA added to cultures 24 or 48 hr after activation produced no significant inhibition indicates that HA does not affect the later activation events leading to replication of these activated cells, such as interactions of IL-2 and its receptor.

The inhibition of lymphocyte proliferation could be due to impairment of IL-2 production, which occurs during the first hours of activation. The amounts of IL-2 in the medium were determined using blasts activated for 6 days with Con A as responding cells. While established mitogen-independent bovine T-cell clones that proliferate in response to an IL-2-like growth factor, are known,[13] there is no evidence that such bovine lines cannot detect T-cell growth factors other than IL-2. Since established IL-2-sensitive murine T-cell clones have been found to detect cytokines other than IL-2,[14,15] we used a titration on heterogeneous blast cells, as described in the Materials and Methods.

PBMC grown in HA plus PHA released less IL-2 activity into the medium than did cells grown in PHA alone, as soon as 24 hr after activation. Addition to the cultures of an anti-IL-2R antibody that blocked the IL-2 interaction with its receptor, potentiated the action of HA in reducing the IL-2 activity in the medium to the same extent. Hence, HA inhibits the IL-2 activity by blocking IL-2 secretion by the PBMC and not by increasing the IL-2 consumption of the cells.

IL-2 production is believed to be an interdependent event that occurs during the interaction of macrophages with T lymphocytes. The macrophage not only initiates the cytokine cascade, but is also believed to down-regulate this cascade by producing PGE_2. This concept is supported by many studies on human and mouse models, which have shown PGE_2 inhibition of IL-2 synthesis by T lymphocytes,[16,17] IL-2-induced T-lymphocyte proliferation[16,18] and interferon-γ synthesis.[19] Therefore, the macrophage may block the production and action of cytokines that normally results in T-lymphocyte, activation by producing PGE_2.[20,21] Prostaglandins have often been associated with parasite-induced immunosuppression, and have been shown to inhibit IL-2 secretion during experimental infections with the related parasites *T. cruzi*,[22] *Leishmania major*[23,24] and *L. donovani*.[25] However, to our knowledge, there has been no report implicating prostaglandins in the inhibition of lymphocyte proliferation and IL-2 production in cattle.[7]

Our data show that the parasite protein, HA, causes the release of PGE_2 from bovine monocytes and PBMC in culture. The cyclo-oxygenase inhibitor indomethacin counteracts the inhibition of the bovine PBMC proliferative response by HA. This suggests that HA is involved in indomethacin-sensitive immunodepression, which then implicates the prostaglandins. On the other hand, an inhibitor of the lipo-oxygenase pathway, NDGA, does not interfere with the HA effect, indicating that leukotrienes play no role in the action of HA.

There is also an overproduction of PGE_2 by monocyte-enriched cultures in response to HA. This observation suggests that HA most probably acts via the release of PGE_2 by activated macrophages. Moreover, the concentration of PGE_2 in the conditioned medium of PHA-activated PBMC grown in HA for 48 hr was significantly higher than without HA. HA may induce a small imbalance in the prostaglandin pathway that leads to immunodepression of the proliferative response. Inhibiting effects of HA may be induced not only by overproduction of PGE_2 by monocytes, but also by abnormal secretion of other molecules in the cyclo-oxygenase pathway, including prostacyclins, thromboxanes or other prostaglandins which could be inhibited by indomethacin. But the role of these cyclo-oxygenase pathway products in the mitogenic proliferative response is not so well documented.

Our study therefore shows that HA inhibits lymphocyte proliferation at the early steps of blastogenesis, and blocks IL-2 production by PBMC in the culture. HA also interacts with monocytes to modulate the prostaglandin pathway, and probably produces a generalized state of immunosuppression in the PBMC cultures that could be associated with down-regulation of IL-2 secretion. HA may also have a direct effect on T lymphocyte or monocyte receptors inducing differentiation, or cytokine receptors or adhesion molecules. These mechanisms remain to be explored.

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