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In vitro Lymphoproliferative Responses of *Trichostrongylus colubriformis* High and Low Responder Guinea Pigs to Worm Antigens (SPL3, SPA) and Ovalbumin

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ABSTRACT

***In vitro* lymphocyte responses of high responder (HR) and low responder (LR) guinea pigs from peripheral blood lymphocytes (PBL) to parasite antigens soluble protein third stage larvae *(SPL3)* and soluble protein adult stage (*SPA),* non-parasite antigen ovalbumin (OVA) were examined. There was substantial differences between HR and LR guinea pigs in the rate of acquisition of responsiveness to these Ags as well as differences in responsiveness to Ags derived from third stage larvae *(SPL3)* and adult worms *(SPA).* Overall, the results suggest both stronger and more rapid acquisition of responsiveness by HR animals and raise the possibility of the animals being able to preferentially respond to larval immunogen and thus acquire protective immunity more rapidly than LR.** *Iran. Biomed. J 2 15-20 (1998)*.

Keywords: Trichostrongylus colubriformis, soluble protein third stage larvae, soluble protein adult stage

INTRODUCTION

In vitro lymphoproliferative responses to Ag and mitogens have been used for many years to assess lymphocyte function and such assessments have been made in several host-parasite systems, including *T. colubriformis* and *Haemonchus contortus (H contortus)* in sheep [1, 2]. The use of *in vitro* lymphocyte proliferation assay as a predictive test for assessing potential resistance of sheep to infection with gastrointestinal nematodes was successful with *H contortus* [3] but results with *T. colubriformis* were not conclusive. Dawkins *et al.* [4] reported that cellular parameters did not differentiate between HR and LR lambs. On the other hand, Win-don and Dineen [1] showed that sheep immune to *T. colubriformis,* had high *in vitro* lymphocyte responses to the *T colubriformis* antigen preparation *SPL3* when compared with susceptible animals. Immunodominant Ags from *T. colubriformis* were also recognized by T- lymphocytes from sheep immunized with excretory-secretory (ES) proteins of the parasite [5].

Although *in vitro* proliferation of lymphocytes from out bred guinea pigs in response to worm Ags has been studied [6], a comparison of these responses in HR and LR guinea pigs has not been made. It was decided to examine *in vitro* responses of lymphocytes from HR and LR guinea pigs to *SPL3* and *SPA.* The purpose of this study was to determine whether the difference in susceptibility of these animals reflected differences in the activation of cells involved in expression of resistance, and in addition, whether *SPL3* and *SPA* had different abilities to induce cell transformations. Because significant differences in responsiveness of HR and LR animals to *T. colubriformis* Ags were found, a similar experiment using OVA (a non-parasite Ag) to immunize guinea pigs was also performed.

MATERIALS AND METHODS

Experimental Design. Guinea pigs used in this work were derived from breeding stock with genetically determined resistance (HR) or suscepti bility (LR) to T *colubriformis* infection. These breeders were outbred and the product of a selective breeding program based on fecal egg counts

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(FEC) following primary infection with *T. colubriformis* as described by Rothwell *et al.* [7]. 4xHR and 4xLR guinea pigs were given standard doses of 2000 *T. colubriformis* third stage larvae *(TcL3)* in 1 ml water by mouth through a metal stomach tube fitted to a 2 ml syringe while anaesthetized by the i.m. injection of xylazine (0.1 mg/100 g) combined with ketamine (3 mg /100 g). Blood was collected before infection and at weekly intervals until 28 d after infection in 5 ml heparinized tubes. PBL were then cultured with parasite Ags, *SPL3* and *SPA* prior to and following infection (Experiment A). FEC were performed as described by Rothwell *et al.* (1994). As a lowest positive egg count of 25 was found in the calculation of FEC, egg per gram (EPG) was transformed to loge (counts +12.5). This transformation tends to be variance stabilizing and to normalize such data. Eight additional guinea pigs (4xHR and 4xLR) were immunized with a single dose of 1 mg OVA dissolved in 1 ml PBS, half being given s.c. and half i.p., then their peripheral lymphocyte responses to OVA measured (Experiment B).

Culture Media. Tissue culture studies were performed in RPMI 1640 supplemented with 10 mM L-glutamine, penicillin-streptomycin (100 units/nil penicillin and 100 mg/ml streptomycin), 39.4 µM 2-mercaptoethanol, and adjusted to pH 7 by 3-4 drops of HCl 1M.

In vitro Lymphocyte Proliferation Assay. A whole blood assay was chosen for measurement of *in vitro* lymphoproliferation. This method has several advantages over the use of separated cells. Of particular importance was the small quantity blood sample required, allowing repetitive sampling of individual guinea pigs. There was also no need to add exogenous serum which could confound the assay results. However, one potential technical problem encountered was the inability to adjust cell densities because blood samples were strictly diluted 1:5 in culture media prior to running the assay. Total leukocyte counts were carried out using gentian violet to address variation in densities. There was a slight difference in cell densities of both lines 7 d after infection, but no significant differences which might have contributed to variability of the responses were found. Cell numbers were arbitrarily set up within ranges between 5×10^4 to 2×10^5 cells per well.

The procedure was carried out as described by Fletcher *et al.* [8] using 96 well flat bottom microtitre plates. Heparinized blood samples were diluted 1:5 in culture medium within 1 h of collection by cardiac puncture and 100 µl dispensed into each well. Optimal dilutions of antigens *(SPL3*-100 µg /ml; *SPA* -100 µg/ml; OVA-100 µg/m1) were added to the cultures in a further 100 µl. Assays were set up in triplicate and single background wells received 100 µl culture medium in place of Ags. Cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 d and pulsed with 1 µCi tritiated thymidine in a volume of 25 µl/well during the last 16 -18 h of the incubation period. At the end of incubation, cultures were harvested onto glass filter paper discs and dried at room temperature overnight. Filter discs were finally placed into plastic bags to which 10 ml of scintillation fluid was added. Radioactivity was counted in a betaplate liquid scintillation counter. Counts of cells in medium alone remained less than 1500 cpm. Proliferative responses were expressed as net geometric mean cpm. In order to exclude negative values, cpm. was transferred to loge (cpm+ x) prior to dependent variable analysis in SAS.

Satisfactory cell stimulation were generally achieved after 3 d incubation plus the 16-18 h tritiated thymidine pulse period and this period was chosen for the experimental work.

Statistical Procedures. Prior to analysis, logarithmic (base e) transformation of the data was performed. All the data are presented as geometric means unless otherwise stated. Ranges of standard error about the mean were also calculated. Either Statistical Analysis System 6.03 [9] or Minitab release 10 (State College USA, 1994) was then used for analysis of variance (dependent variable and Two-way ANOVA), and Student's t-test or Student-Newman-Keels test were used for further analysis. In experiments where sequential samples were taken from the same animals, split-plot-in-time analysis of variance was used. Further comparisons of group means were also carried out on the data output using l.s.d [10]. Significance was indicated where $P < 0.05$. There was no significant sex difference in all the experiments carried out in this study.

RESULTS

Fecal Egg Counts. Geometric mean FEC of these HR and LR guinea pigs during challenge with *T colubriformis* are depicted in Figure 1.

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Throughout the period of observation, LR guinea pigs showed higher egg counts than HR animals (P<0.001). In LRs, EPG reached a peak on day 23 (mean 2331, Fig. 1), thereafter fell to 1391 and 998 by days 25 and 28 respectively. HRs had a mean counts of 49 EPG on day 16 and no eggs we found in the feces after day 21.

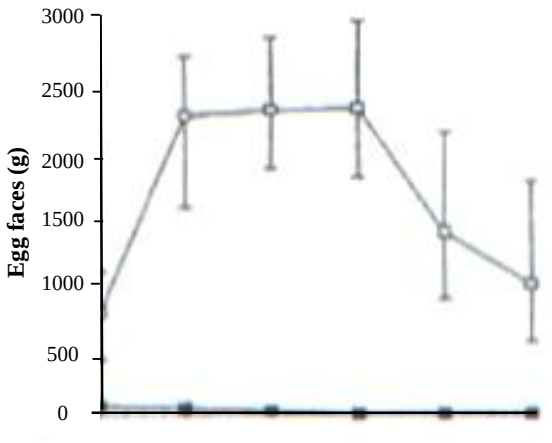

Fig. 1. Eggs per gram faeces (geometric mean) of 4xHR and 4xLR guinea pigs following infection with 2000 infective larvae of *T colubriformis.* ■ high responder (HR). low responder (LR).

Responses to Antigen (Experiment A). Time courses of proliferative responses of PBL from HR and LR guinea pigs incubated with parasite Ags are shown in Figure 2. Poor blastogenic responses to *SPL3* and to *SPA* were observed in both lines prior to experimental infection, but significant proliferative responses developed following infection. Blastogenic response to *SPL3* was greater in HRs than in LRs following infection *(P* < 0.05). HR animals responded to *SPL3* after primary infection, reaching mean cpm of 1972 v 106 (Figure 2). The peak response was detected on day 21 then fell by day 28. LR animals, however, showed a suppression in response to *SPL3* on day *7 (P* < 0.05) but positive responses developed as the infection progressed.

Proliferative responses to *SPA* in both lines did not show a significant change until day 7 of infection, and reached their peak on or after day 28. Split plot in time analysis revealed similar responses of both HR and LR animals to *SPA* following infection. However, the only between lines significant difference detected was on day 21 when HRs were more responsive than LRs (mean 16528 v 2852, Fig. 2).

Comparison of responses to the two Ags by means of split-plot in time analysis during primary infection showed similar responses in HRs to both Ags, whereas LRs were more responsive to *SPA* than to *SPL3* (1511 v 383, *P* < 0.05 Fig. 3).

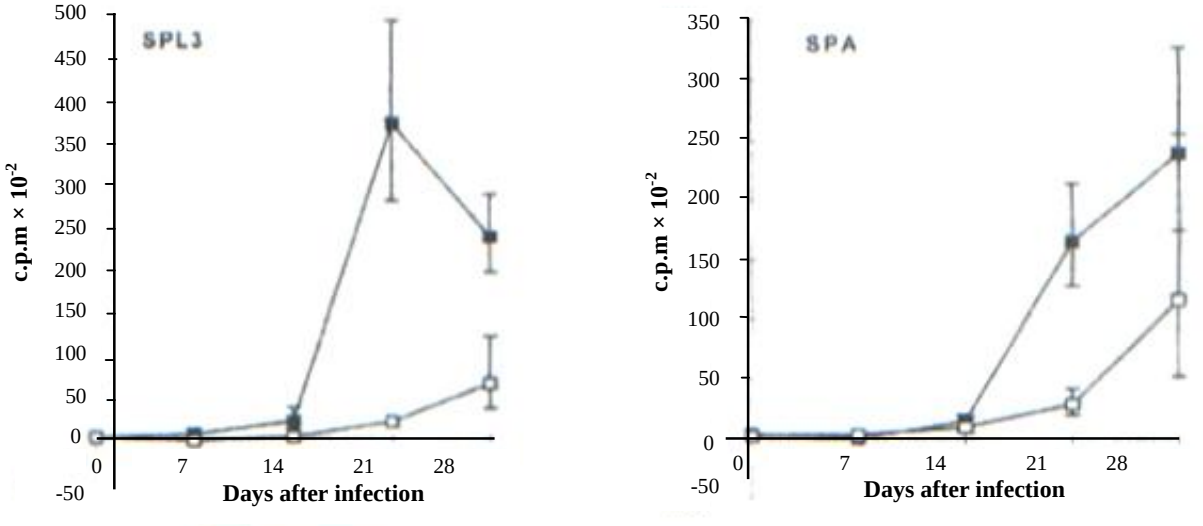

Fig. 2. Proliferative responses (geometric mean c.p.m.) of PBL from 4xHR and 4xLR guinea pigs to *T. colubriformis* antigens *(SPL3, SPA)* following infection. ■ high responder (HR), low responder (LR).

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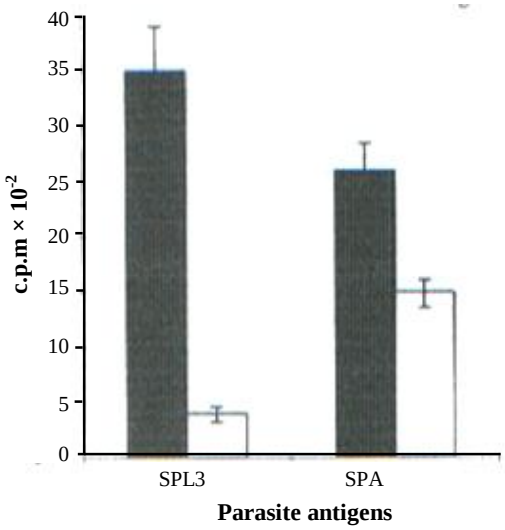

Fig. 3. Pooled proliferative responses of PBL (geometric mean c.p.m.) from 4xHR and 4xLR guinea pigs to SPL3 and SPA during primary infection with 2000 *T. colubriformis* infective larvae. ■ high responder (HR), low responder (LR).

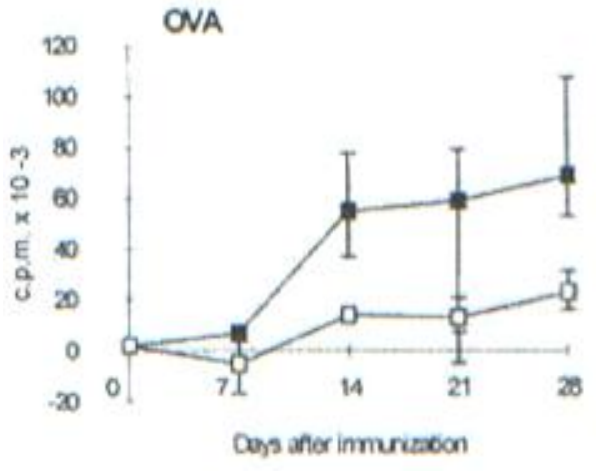

Fig. 4. Proliferative responses (geometric mean c.p.m.) of PBL from 4HHA. and 4HLR guinea pigs to non-parasite antigen (OVA) following immunization with 1 mg ovalbumin. P high responder (HR), R low responder (LR).

Non-parasite Antigen (Experiment B). *In vitro* lymphocyte proliferative responses of OVA- immunized guinea pigs to OVA are depicted in Fig. 4. Both lines responded to OVA following immunization *(P*= 0.0017). Although there was a significant difference between the lines at all data points *(P*= 0.04). Split plot in time analysis revealed that this difference was due to a difference on day 7, in which LR animals showed depressed responses to OVA. Both lines also peaked on 28 d after immunization (mean cpm. of HR = 6888 and LR = 2315, Fig. 4).

DISCUSSION

This study demonstrates that the whole blood culture technique is satisfactory for lymphocyte proliferation assays in guinea pigs. Optimum stimulation time was generally 3 d at 37 °C plus 16-18 h methyl- ^{3}H-thymidine pulse time. Zimmerman *et al.* [11] reported a cell density of 8×10^4 cells per well to be optimum for ovine lymphocyte cultures using the whole blood technique. This density was within the ranges used in present study. Both lines revealed slight, but statistically insignificant increases in total leukocyte counts following infection that could be due to *in vivo* proliferation of lymphocytes to *T. colubriformis* infection.

The results demonstrated that PBL from both lines of guinea pigs acquired responsiveness to parasite Ags following infection with *T. colubriformis.* They also demonstrate substantial differences between HR and LR guinea pigs in the rate of acquisition of responsiveness to these Ags as well as differences in responsiveness to Ags derived from third stage larvae *(SPL3)* and adult worms *(SPA).* Further, responses to OVA were generally greater in HR animals and finally, in response to both parasite Ags and OVA, circulating lymphocytes from LR animals generally showed lower responsiveness when examined from 7 d after infection or immunization. However, it was previously reported (unpublished data) that parasite-infected and OVA-immunized guinea pigs revealed different isotype-specific antibody responses and significant difference between the lines observed following infection only. Overall, the results suggest both stronger and more rapid acquisition of responsiveness by HR animals and raise the possibility of the animals being able to preferentially respond to larval immunogen and thus acquire protective immunity more rapidly than LR.

This study supports the finding of Dobson and Soulsby [6] who reported peak proliferative responses of outbred guinea pigs to *T. colubriformis* Ags 25 d after infection. Of particular relevance is the observation of McClure *et al.* [12] that HR sheep responded to *SPL3* stronger and earlier than LR sheep and that their responsiveness to *SPL3* was stronger than their responsiveness to *SPA.* The different capacity of *SPL3* and *SPA* to stimulate HR and LR lymphocytes, suggests that a variety of Ags in each of these preparations provoke immune responses in the host. Such Ags might be common between larvae and adults or there might be quantitative or qualitative differences between the Ags in the different parasite stages. For example, a 94

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kDa glycoprotein from *TcL3* [13], 18 kDa and 30 kDa from *TcA* [14, 15] and a 41 kDa from both *TcL3* and *TcA* (16) have been reported to be capable of partially protecting hosts against *T. colubriformis.* Thus, *SPL3* may contain a greater number of Ags with stronger abilities than those in *SPA* to elicit protective immune responses in guinea pigs. This may account for inverse correlation between FEC and increasing cell transformation by *SPL3* (days 14 and 21) and *SPA* (day 21) where peak proliferation occurred at the time worm eggs disappeared from the feces of HR guinea pigs (Fig. 1).

Despite similar antibody responses of both lines to OVA (unpublished data), PBL from HR animals responded to OVA earlier than LR animals. Split plot in time analysis revealed that LRs exhibited significantly depressed responsiveness (cpm) on day 7. Faster responses to OVA in HRs (day 7 to 14) than in LRs (day 21 to 28) may be due to enhanced Ag presentation to T-cells of HRs. Lutje and Black [17] reported that OVA-specific *in vitro* responses of *in vivo* primed PBL were dependent on the presence of $CD4^+$ T-cells to which OVA was presented in a MHC II restricted manner. Therefore, LRs might be genetically less capable of MIIC II surface molecule expression following foreign Ag activation as they revealed lower cpm than HRs during immunization. Further, B-cells of guinea pigs contain considerably larger amounts of MHC II products than do T-cells [18] and they are capable of presenting Ag to primed T-cells in a MHC-restricted fashion [19, 20]. Neither macrophages nor dendritic cells can efficiently take up soluble Ags, but B cells are uniquely adapted to bind soluble molecules through their cell surface Ig. Both OVA and *SPL3* are soluble proteins to which CD4 T cell responses require B cells as APC [21]. Thus, the relatively poor responses of LR guinea pigs to OVA might also be due to a lower frequency of B-cells [22] leading to a poorer ability in Ag presentation to $CD4^+$ T-cells. This hypothesis was reinforced by higher (P=0.0005) ability of HRs to respond to PWM prior to and following immunization with OVA (unpublished data).

LR guinea pigs eventually develop resistance to *T. colubriformis* after termination of their primary infection. Their stronger response to *SPA* suggests that their less effective and delayed protective responses compared with HR animals may be due to their different profile of Ag recognition. Further, preliminary experiments on multiple-infected guinea pigs showed that LRs were more responsive to *SPA* than to *SPL3* whereas HRs showed similar responses to both *SPA* and *SPL3* (data not shown). Again, higher responses of HR animals to both Ags may be due to different immunodominant proteins recognized by HRs and LRs.

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