

Screening for immunomodulatory proteins of the intestinal parasitic nematode *Heligmosomoides polygyrus*

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SUMMARY

Parasitic nematodes are constantly exposed to the immune effector mechanisms of their hosts. One strategy of the worms to cope with these defence reactions is the secretion of modulatory proteins that down-regulate cell-mediated immune responses. We analysed the proliferation of mesenteric lymph node cells of mice infected with *Heligmosomoides polygyrus* and showed that cellular proliferation was strongly suppressed in the chronic phase of infection. To identify proteins of *H. polygyrus* that are involved in parasite-induced immunomodulation, worm extract and culture supernatant of adult *H. polygyrus* were fractionated by gel chromatography and activity of each fraction was determined. One of the fractions (fraction 9) of worm extract as well as worm secretory products inhibited the antigen-specific cellular proliferation by about 40%. This reduced cellular reactivity coincided with a down-regulation of inducible nitric oxide production of mouse macrophages by 57%. Furthermore, fraction 9 contained antigens that were recognized by IgE antibodies of *H. polygyrus*-infected mice and induced degranulation of an IgE-sensitized basophil cell line. Single proteins of fraction 9 were analysed by mass spectrometry. These data suggest that antigens that are recognised by IgE antibodies might play an important role in immunomodulation exerted by nematode infections.

Keywords allergen, cellular hyporeactivity, mass spectrometry, parasitic nematode

Abbreviations RBL: Rat basophil leukaemia cell line

INTRODUCTION

Infections of humans and other animals by parasitic gastrointestinal nematodes are the cause of several widespread diseases. Such infections are frequently long lasting, and recent data have shown that highly effective immune evasion mechanisms of the parasites are a key element of this persistence (1–3). The typical immune response generated by a primary infection with *Heligmosomoides polygyrus*, a nematode that dwells in the duodenum of the mouse for more than 8 months (4), is characterized by strongly up-regulated Th2 responses. This results in elevated production of IL-4, tissue eosinophilia, mastocytosis, goblet cell hyperplasia and parasite-specific IgE, IgA and IgG1 responses. However, prolonged primary infection is also associated with the down-regulation of certain components of the Th2 responses, including IL-9, IL-10 (5), IL-13 (6,7) and intestinal mastocytosis (8). IL-4 is an absolute requirement for the expulsion and protective immunity to *H. polygyrus* (9,10), whereas IL-13 does not appear to be essential. Host protective immunity against *H. polygyrus* can be induced by drug-abbreviated primary infection or by multiple infections (7).

Resistance to *H. polygyrus* infection of different mouse strains is, as in the case of other gastrointestinal nematode infections, Th2-dependent and was described as associated with chromosomal regions influencing Th2 activity and the level of Th2-dependent effector activities (11,12). Variations in the degree of resistance reflect differences in immune and inflammatory responses. These are associated with serum levels of mucosal mast cell proteases that are indicative of mast cell activation or elevated serum IgE levels (13). In addition, it has recently been shown that changes of the intestinal metabolism, such as an increase in the rate of epithelial turnover, which is under the control of IL-13 and the chemokine CXCL10, act to expel gastrointestinal nematodes (14). Interestingly, a strong Th2 response occurs in all strains of mice that are infected with *H. polygyrus*. However, many strains fail to expel *H. polygyrus*. Therefore, it was

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Received: 11 August 2005

Accepted for publication: 10 April 2006

hypothesized that the Th2 response in the susceptible or intermediate strains occurs in a modified form or that modulating mechanisms such as regulatory T cells intervene to prevent worm expulsion (1).

Despite a relative wealth of information on immunoregulation in nematode infections, relatively few modulatory proteins have been identified so far (15,16). Infection with *H. polygyrus* might lend itself to the identification of such molecules, because these worms not only impair homologous immunity (17), but also efficiently suppress immune responses against a variety of unrelated antigens, such as sheep red blood cells (18), or against other nematodes such as *Trichinella spiralis* (19). In addition, immunosuppressive activity was also shown *in vitro* by secreted products of adult worms (20,21). One secreted factor described as an immunomodulator of *H. polygyrus* is the 'immunomodulatory factor' (IMF). IMF was shown to inhibit the production of antibodies. It was suggested that the effect of IMF is mediated by T lymphocytes, because T cell-depleted cultures failed to respond to IMF and the percentage of CD8⁺ T cells increased in IMF-treated cell cultures (22,23). However, although the molecular nature of *H. polygyrus* IMF is not yet known, it appears to be of a non-proteinaceous nature (23).

In this paper, we used infection with *H. polygyrus* as a rodent model to identify immunomodulatory nematode products and investigated the immunosuppressive activity in a heterologous model, using spleen cells of OVA-TCR-transgenic mice. We describe the identification of an IgE-recognized protein fraction of *H. polygyrus* extracts and E/S-products obtained by gel chromatography that down-regulated antigen-stimulated cellular proliferation.

MATERIALS AND METHODS

The parasite

Heligmosomoides polygyrus bakeri was maintained by serial passage in BALB/c mice. Infective larvae (L3) were obtained from faecal culture. Mice were infected orally with 120 L3 with the use of a feeding tube.

Preparation of *H. polygyrus* homogenate and production of *H. polygyrus* excretory/secretory products

Adult worms were collected from mouse intestine approximately 21 days after infection, and 50 worms were cultured in 500 µL of RPMI medium (Biochrom, Berlin, Germany) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM) for 24 h in 37°C, 5% CO₂. Supernatant containing excretory/secretory products of the nematode was collected and concentrated by centri-

con columns (Vivascience, Hannover Germany). The worms (about 300 male and female adults) were sonicated in 0.5 mL PBS (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and centrifuged. Supernatant was collected and filtered sterile, and protein concentration was measured by the BCA test (Pierce/Perbio, Bonn, Germany).

Superdex-200HR gel filtration of *H. polygyrus* antigens

Separation of nematode antigens was carried out using FPLC-chromatography (Amersham). A Superdex-200 column was used to separate antigens according to their molecular size. The antigen was eluted isocratically with PBS (pH 7.4) using the flow rate of 0.4 mL/min. Fractions of 1 mL volume each were collected over a period of 88 min, filtered and stored at -20°C until use. The molecular weight of each single fraction was calculated according to a standard curve. The protein concentration of each antigen fraction was calculated by the BCA test (Pierce/Perbio, Bonn, Germany). Fractionation of somatic antigen was carried out with four different antigen preparations from about 200 freshly isolated worms each. The chromatogram and the SDS-PAGE shown are typical for four independent fractionations.

Proliferation of mesenteric lymph node (MLN) lymphocytes from *H. polygyrus*-infected mice

BALB/c mice were infected with 120 L3 of *H. polygyrus*. At 2 and 4 weeks after infection, three BALB/c mice were sacrificed and MLNs were isolated aseptically. Single-cell suspensions were obtained from pooled MLNs and resuspended in RPMI (Biochrom, Berlin, Germany) supplemented with 5% foetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM L-glutamine. 3.5×10^5 cells per well were plated in 96-well flat-bottom culture tissue plates (Costar) and incubated in triplicate with 12 µg/mL of *H. polygyrus* somatic extract or 5 µg/mL concanavalin A (ConA; Sigma) or without stimulus. Two independent experiments were carried out.

Antigen-driven proliferation of murine spleen cells

Spleens of three male OVA-TCR-transgenic (24) mice (purchased from BfR, Berlin, Germany) were removed aseptically, minced and passed through a stainless steel mesh to obtain single-cell suspensions. The cells were depleted of erythrocytes using NH₄Cl lysis, washed and resuspended in RPMI (Biochrom, Berlin, Germany) supplemented with 5% foetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL) and 2 mM L-glutamine. 3.5×10^5 spleen cells per well were plated in 96-well, flat-bottom tissue culture plates, and lymphocyte proliferation was induced with

ovalbumin (OVA, 40 μ M) (Sigma, Taufkirchen Germany). CD4 T cells of OVA-TCR-transgenic mice recognize a T cell epitope spanning amino acids (aa) 323–339 of chicken ovalbumin in the context of MHC class II. The spleen cells were stimulated with ovalbumin and 40 μ L of each HPLC-separated fraction were added. The final volume of each well was 200 μ L. Synthesis of DNA was estimated by incorporation of [^{3} H]thymidine (1 μ Ci/well; Amersham Pharmacia Biotech, Gent, Belgium), which was added during the last 20 h of a 72 h culture. Experiments were performed in triplicate and the data are presented as mean $\pm$ SD. The experiment shown is representative of three independent experiments.

Nitric oxide (NO) production of murine peritoneal macrophages

Resident peritoneal macrophages were harvested by washing the peritoneal cavity of six male BALB/c mice with RPMI (without FCS). Cells were pooled, depleted of erythrocytes by NH_4Cl lysis, washed and resuspended in RPMI (Biochrom, Berlin, Germany) supplemented with 5% foetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 μ g/mL), and 2 mM L-glutamine. The viability of cells was assessed by trypan blue exclusion, and cells were plated in 96-well flat-bottom tissue culture plates (Costar) at a density of 2×10^5 cells per well, and allowed to adhere to the microtitre plate for 2 h under standard cell culture conditions. Non-adherent cells were removed from the plates and resting cells were activated with recombinant murine (rm) IFN- γ (100 U/mL Tebu, Frankfurt, Germany). The activated cells were incubated for 24 h in the presence of 40 μ L of each FPLC-separated *H. polygyrus* antigen fraction in a final volume of 300 μ L. NO production was assayed by measuring nitrite by the Griess reaction according to Green *et al.* (25). Briefly, 100 μ L aliquots of the cell culture supernatant were mixed with an equal volume of Griess reagent [1% sulfanilamide (Sigma), 0.1% naphthylethylene diamine dihydrochloride (Sigma), 2% H_3PO_4 (Merck)] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader. NO_2^- was quantified using 100 μ L NaNO_2^- of different concentrations as a standard. Experiments were performed in triplicate and data are presented as mean $\pm$ SD. The experiment shown is representative of three independent experiments.

Rat basophil leukaemia (RBL) cell mediator release assay

IgE-binding activity of proteins was tested using an *in vitro* allergen assay based on the release of hexosaminidase by RBL cells, which are sensitized by IgE (26). RBL cells were maintained in Eagle's minimum essential medium with 20% RPMI, 5% foetal calf serum (FCS), penicillin (100 U/mL),

streptomycin (100 μ g/mL) and L-glutamine (2 mM). Cells were harvested in the stationary phase and plated in 96-well flat-bottom plates (10^5 cells/well). Cells attached to the surface during 8–12 h of incubation. Passive sensitization of the cells was accomplished by 1 h incubation with serum from *H. polygyrus*-infected mice diluted 1:50 in the medium. Subsequently, cells were washed three times with Tyrode's buffer (Sigma). For cross-linking of the surface IgEs, RBL cells were co-cultured with 40 μ L of antigen fractions diluted in 60 μ L Tyrode's buffer for 1 h. Spontaneous and total release were measured by incubation of cells with Tyrode's buffer without antigen, and with addition of 1% Triton X-100 (Serva), respectively. For measurement of β -hexosaminidase, 30 μ L of supernatant was transferred into wells of another plate containing 50 μ L of p-nitrophenyl N-acetyl β -d-glucosaminide (Sigma) as a substrate (1.3 mg/mL in 0.1 M citric buffer, pH 4.5). The reaction was terminated by the addition of 100 μ L of 0.2 M glycine solution pH 10.7. Absorbance was measured at 405 nm. Results are expressed as percentage of the total release after subtraction of spontaneous release. The assays were conducted in triplicate and data are presented as mean $\pm$ SD. The experiment shown is representative of two independent experiments.

SDS-PAGE and mass spectrometry

SDS-PAGE was performed according to the method of Laemmli. Samples were suspended in loading buffer, boiled for 5 min, and electrophoresed at 20 mA on a 10% or 14% (wt/vol) polyacrylamide gel. Proteins were visualized by Coomassie Brilliant Blue staining. For further protein analyses, the bands of interest were excised, digested, and purified as previously described (27). For protein analyses, high-pressure liquid chromatography (HPLC) coupled to the mass spectrometer (Qstar XL, Applied Biosystems) was used, and automated MS/MS fragmentation was performed during the HPLC run. Peptide sequences were determined manually by using Analyst QS Bioanalyst extensions (Applied Biosystems) from fragmentation spectra and accomplished with database searches (NCBI).

Statistical analysis

Data are presented as means $\pm$ SD. Experiments shown are representative of at least two independent experiments. Statistical analysis was performed using the program SPSS 13 for Windows. In respect of Figure 1, the mean differences were tested using a split-plot design with a mixed-model approach (28). Here, two independent experiments were analysed with two fixed factors (time: 2 and 4 weeks and treatment: RPMI, ConA and *H. polygyrus* antigen) and one random factor (pool of cell suspension). Within this

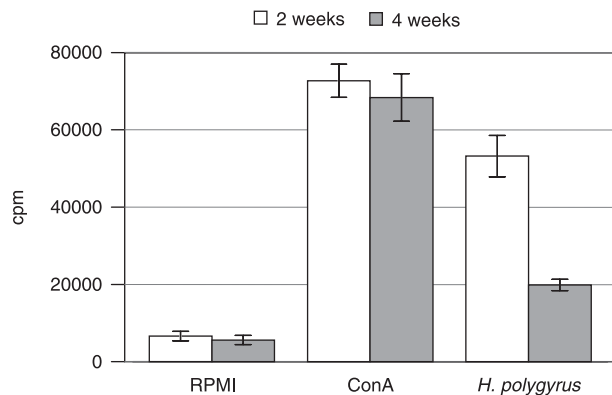


Figure 1 T cell proliferation of mesenteric lymph node cells (MLN) of BALB/c mice from 2 and 4 weeks after *H. polygyrus* infection. Cellular proliferation was measured in the presence of ConA or somatic antigen of *H. polygyrus*. Proliferation of cells was determined by incorporation of [^{3} H]thymidine which was added during the last 20 h of the culture. The experiments were conducted in triplicate and data are presented as means $\pm$ SDs. The data shown originate from two independent experiments. ConA: concanavalin A, RPMI: cell culture medium.

model the random factor 'pool' was fixed under the factor 'time period' and also the interaction of both fixed factors was included. We used data from four sources of pooled cells from three mice each: one pool of cells from the first experiment after 2 weeks, a second pool of cells from the first experiment after 4 weeks and two further pools of cells from the second experiment. Within each pool three treatments were applied, each with three independent replicates. Having found significant differences, Sidak's *post hoc* test was used together with contrast test (*t*-test) that considered only the interaction term. With regard to Figures 3 and 4, a one-way ANOVA of fractions 0–20 on proliferative response of cells, $n = 3$ per fraction, was used. Having found significant overall differences, Dunnett's *post hoc* test for comparison of different groups with a control was used to calculate which groups are significantly different from the control group. With respect to Figure 5 a one-way ANOVA of the reactive fractions 6–16 on their degree of degranulation, $n = 3$ for each fraction, was used. Having found significant overall differences, Tukey's HSD *post hoc* test was used, which verifies simultaneously the differences between each pair of means. The *P* values, which are generated from *post hoc* tests, are not accompanied by *F* values.

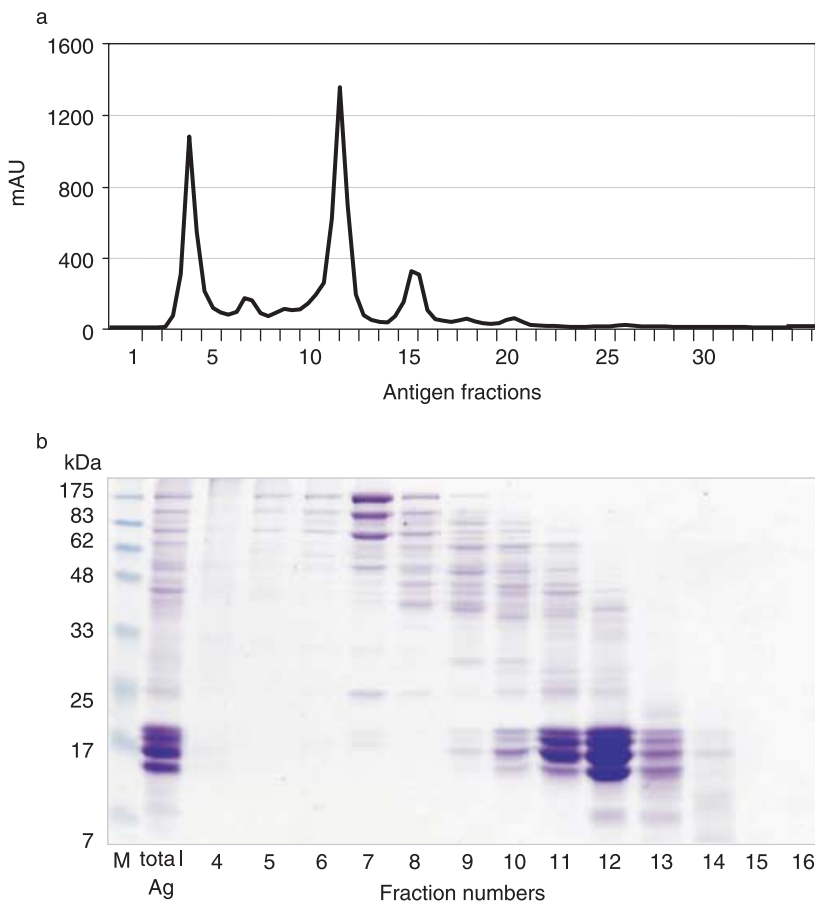


Figure 2 (a) FPLC-gel chromatogram of *H. polygyrus* somatic antigen. A superdex-200 column was used to separate proteins according to their molecular size. The antigen samples were eluted isocratically with PBS (pH 7.4) using a flow rate of 0.4 mL/min. 35 fractions of 1 mL volume each were collected. (b) SDS-PAGE (14%) of *H. polygyrus* separated antigen fractions. 100 μ L of each fraction was precipitated by TCA and analysed under reducing SDS-PAGE conditions. M: molecular weight marker.

Figure 3 Antigen-induced proliferation of spleen cells of OVA-TCR-transgenic mice in presence of separated antigen fractions. The cells were cultured with 40 μ L of each fraction of *H. polygyrus* somatic antigen (a) or 40 μ L of five-fold concentrated fraction from the nematode's excretory-secretory products (b). Fraction 0 shows the value of OVA-stimulated cells without addition of *H. polygyrus* antigen. The cellular proliferation was determined by incorporation of [^{3} H]thymidine, which was added during the last 20 h of the culture. Experiments were conducted in triplicate and data are presented as means $\pm$ SDs. The experiment shown is representative of three independent experiments.

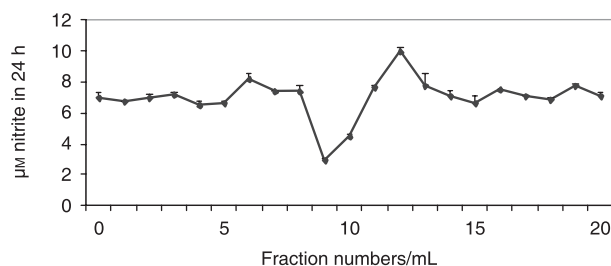
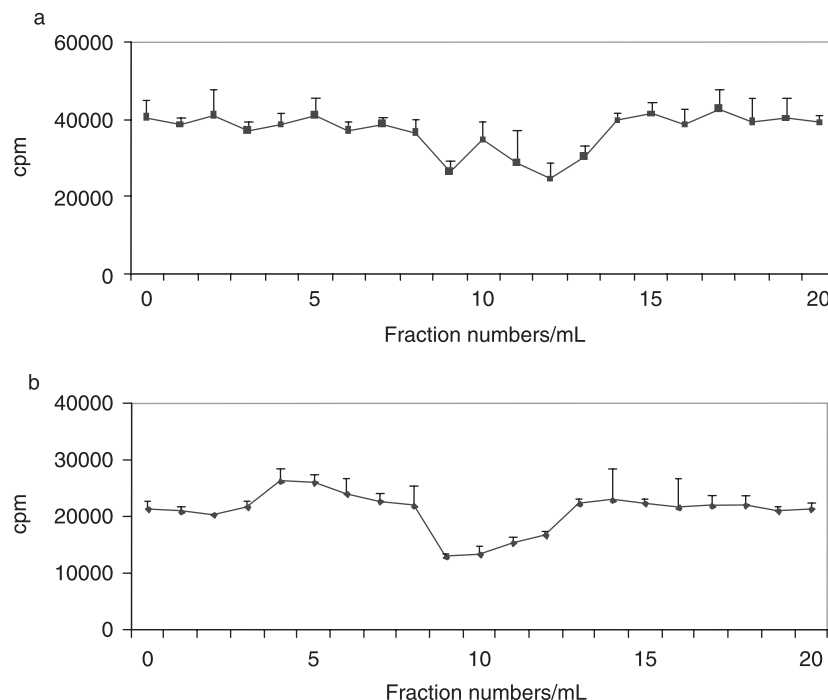


Figure 4 Inhibition of nitric oxide production by fraction 9 of somatic antigen of *H. polygyrus*. NO release of IFN- γ -stimulated mouse peritoneal macrophages co-cultured with 40 μ L of each antigen fraction was determined by measuring nitrite via the Griess reaction. Fraction 0 shows the value of IFN- γ -stimulated cells without addition of *H. polygyrus* antigen. Experiments were conducted in triplicate and data are presented as means $\pm$ SDs. The experiment shown is representative of three independent experiments.

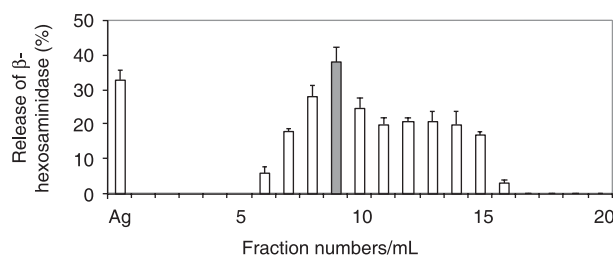


Figure 5 Allergenic capacity of fractionated *H. polygyrus* antigen. IgE antibodies of a serum pool of five *H. polygyrus*-infected BALB/c mice were used to sensitize RBL cells. For cross-linking, RBL cells were co-cultured with 40 μ L of each antigen fraction. Results are expressed as percentage of release of β -hexosaminidase of sensitized RBL cells after subtraction of the spontaneous release. The assay was conducted in triplicate determinations. Means and SDs are shown. The experiment shown is representative of two independent experiments.

RESULTS

Cellular proliferation of mesenteric lymph node cells of infected mice

Mesenteric lymph node cells of *H. polygyrus*-infected BALB/c mice were harvested 2 and 4 weeks post infection. Stimulation of cells as well as the different time points post infection and the interaction of stimulation and time had a significant effect on the proliferation of the cells (Figure 1,

weeks: $F_{1,2} = 40.7$ ($P = 0.024$); treatment: $F_{2,28} = 921.8$ ($P < 0.001$); week $\times$ treatment: $F_{2,28} = 70.4$ ($P < 0.001$) using the split-plot design (28). A multiple comparison between all pairs of treatment showed highly significant differences ($P < 0.001$) using a Sidak's *post hoc* adjustment that is not accompanied by F values. The reduction of proliferation between the two points of time for the antigen-specific stimulus (12 μ g/mL adult worm antigen) is significantly stronger ($t = -9.2$ ($P < 0.001$) with 30 degrees of freedom) in comparison to the reduction of proliferation after ConA stimulation tested by an appropriate contrast test.

Fractionation of somatic antigen of *H. polygyrus*

A total of 250 µL of adult homogenate (~1 mg protein) was applied to a Superdex-200HR column. The fractionation yielded 35 fractions of 1 mL volume each. Proteins of different molecular weights were detected in fractions 4–16 by measuring absorbance at 280 nm (Figure 2a). The concentrations of proteins within the fractions varied from 5 µg/mL to 175 µg/mL, as estimated by the BCA test. Figure 2b shows an SDS-PAGE (14%) under reducing conditions of 100 µL of each fraction, after precipitation with TCA. *Heligmosomoides polygyrus* proteins of molecular weights between 175 kDa and 6 kDa were detectable in fractions 5–14.

Effect of separated *H. polygyrus* proteins on cellular proliferation

To determine whether the separated *H. polygyrus* proteins interfered with the antigen-specific T cell proliferation, we used splenocytes from OVA-TCR-transgenic mice, which specifically respond to the OVA³²³⁻²²⁹ T cell epitope. These cells were incubated with 40 µL of each fraction and stimulated antigen-specifically with OVA. The one-way ANOVA of fractions 0–20 on proliferative responses of OVA-stimulated lymphocytes, $n = 3$ per fraction, showed significant overall differences ($F_{20,42} = 5.66$, $P < 0.001$). The inhibitory effects of particular fractions were analysed using Dunnett's *post hoc* test for comparison with a control (here, the P values are not accompanied by F values). Four fractions: 9, 11, 12 and 13, containing proteins from 160 kDa to 17 kDa, showed immunosuppressive activity (Figure 3a). Fraction 9 (protein content: 8 µg/mL) decreased the proliferation of OVA-stimulated cells by 35% ($P = 0.001$) in comparison to OVA-treated cells. Fractions 11, 12 and 13 also down-regulated cellular proliferation by 29% ($P = 0.007$), 39% ($P < 0.001$) and 25% ($P = 0.03$), respectively, (Figure 3a), but these fractions contained higher concentrations of proteins (up to 36 µg/mL). After adjusting the protein content of fraction 12 to 8 µg/mL only a small and not statistically significant inhibitory effect on T cell proliferation (15%) was found, whereas fraction 9 decreased proliferation by 40%.

We then analysed the effects of the separated E/S products of *H. polygyrus* on lymphocyte proliferation. Overall changes induced by E/S fractions (fractions 0–20) were found to be significant ($F_{20,42} = 9.28$, $P < 0.001$). The subsequent *post hoc* test by Dunnett showed that fractions 9 and 10 had a strong inhibitory effect on the proliferation of OVA-stimulated splenocytes (Figure 3b, the P values of the *post hoc* test are not accompanied by F values). The inhibition of antigen-specific T cell proliferation induced in the presence of E/S molecules of fraction 9 was 39% ($P < 0.001$) and that of fraction 10 was 38% ($P < 0.001$). Fraction 11 also

suppressed proliferation of OVA-stimulated lymphocytes by 28% ($P = 0.009$). However, the protein concentration of fraction 11 was 3–4 times higher in comparison to fraction 9 or 10. The protein concentration of fractions 9 and 10 from ES products was approximately 20 times lower (0.4 µg/mL) in comparison to the protein concentration of fraction 9 or 10 from somatic antigen.

Influence of separated *H. polygyrus* proteins on inducible nitric oxide production of macrophages

We investigated the effect of *H. polygyrus* proteins on the nitric oxide (NO) production of IFN- γ -stimulated mouse macrophages, and overall changes induced by the fractions were found to be significant ($F_{20,42} = 74.62$, $P < 0.001$, Figure 4). 24-h culture supernatants of IFN- γ -activated macrophages contained 7 ± 0.05 µM nitrite, whereas no nitrite was detected in the supernatants of non-activated cells. The addition of 40 µL of *H. polygyrus* somatic extract fractions significantly altered the inducible NO production of activated macrophages. Fractions 9 and 10 showed a significant down-regulation of the nitrite production in the culture supernatant by 57% ($P < 0.001$) and 35% ($P < 0.001$), respectively, using Dunnett's *post hoc* test in which the P values are not accompanied by F values. In addition, reproducible up-regulation of NO by 43% was observed after addition of fraction 12 ($P < 0.001$). Fractions 11 and 13 slightly enhanced the NO level of activated macrophages ($P = 0.023$ for fraction 11 and $P = 0.010$ for fraction 13). Other fractions did not show an effect on NO production.

Recognition of separated *H. polygyrus* proteins by IgE

To investigate the IgE-binding reactivity of *H. polygyrus* somatic antigen, RBL cells were passively sensitized with pooled sera of five *H. polygyrus*-infected BALB/c mice. Sera were collected 2 weeks post infection and used at a dilution of 1 : 50. Degranulation of sensitized RBL cells was induced by the cross-linking of IgE antibodies bound by the Fc-receptors of the cells with 40 µL of each fraction or 10 µg/mL of total nematode extract (Figure 5). The stimulation of RBL cells with total antigen led to a degranulation rate of RBL cells of 34%. Eleven fractions (fractions 6–16) of somatic extracts of *H. polygyrus* led sensitized RBL cells to degranulate. The overall differences of means between all reactive fractions were found to be significant ($F_{10,22} = 31.54$, $P < 0.001$). A *post hoc* comparison of all reactive fractions showed that the degranulation induced by fraction 9 was significantly higher in comparison to all other fractions ($P < 0.001$ calculated by Dunnett's *post hoc* test in which the P values are not accompanied by F values).

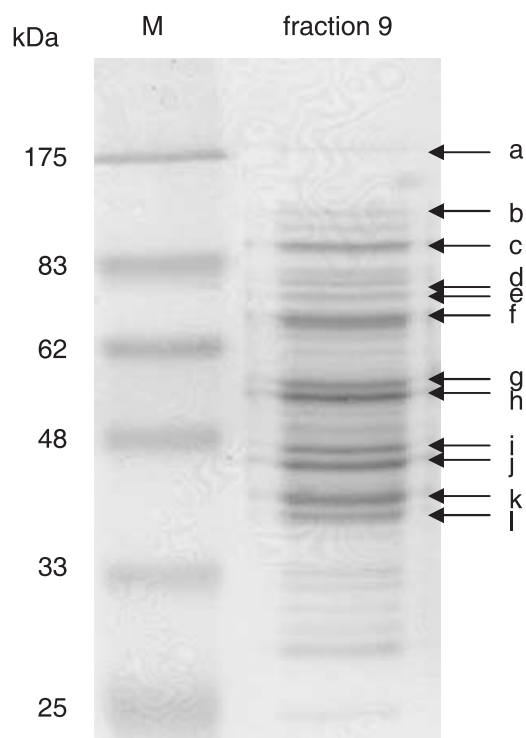


Figure 6 SDS-PAGE analysis of the reactive fraction 9 of separated somatic antigen of *H. polygyrus*. Twelve distinguishable protein bands (a to l) were excised and peptide sequences were analysed by mass spectrometry.

Identification of single proteins in fraction 9 by mass spectrometry

The protein profile of fraction 9 of the somatic antigen of *H. polygyrus* obtained upon reducing SDS-PAGE (10%)

and Coomassie staining is shown in Figure 6. The immunomodulatory fraction 9 comprised at least 15 visible protein bands of molecular weights (MW) from 175 to 30 kDa. Twelve protein bands were excised from the stained gel and analysed by mass spectrometry. Seven of the analysed proteins showed homologies to known proteins (Table 1). All homologous proteins determined were of helminth origin. One protein migrated on SDS-PAGE at a MW of 74 kDa (Table 1, e). This protein was found to exhibit homologies to a 70 kDa heat shock protein of *Caenorhabditis elegans* (gi|123646). Seven peptides with a total length of 60 amino acids matched with the 70 kDa heat shock protein. A second protein that runs on SDS-PAGE at a MW of 70 kDa (Table 1, f) was found to show homology to another heat shock protein (gi|17541098) of *C. elegans*. Here, nine peptides with 130 amino acids in total were found to match to the 70 kDa protein of *C. elegans*. One protein with a molecular weight about 55 kDa (Table 1, g) showed homology to disulphide isomerase from *Ostertagia ostertagi* (gi|16945685). Here, eight peptides comprising 117 amino acids in total were found to be similar to the disulphide isomerase. Another protein migrating on SDS-PAGE at the mass of 50 kDa was determined to show homology to a calreticulin-like protein of *Haemonchus contortus* (gi|4117449). Three matching peptides with a total length of 26 amino acids were determined (Table 1, h). Additionally, a protein of about 46 kDa (Table 1, i) was found with homology to the *C. elegans* calumenin-like protein precursor (gi|17568995). Here, three matching peptides with total length of 34 amino acids were determined. One protein of a molecular weight of 40 kDa showed homology to fructose-bisphosphate aldolase (Table 1, k) from *Echinococcus granulosus* (one matching peptide of 11 amino acids; gi|29336561)

Table 1 Homologies of peptide sequences of proteins from fraction 9. Seven proteins of fraction 9 showed homologies to known proteins. The sequences of the peptides which matched to the *H. polygyrus* protein bands are shown.

Proteins of fraction 9	Homologies within the database	Sequences of matched peptides
(e)	Heat shock 70 kDa protein <i>C. elegans</i>	VEIANDQGNR; DAGTIAGLNVVR; VMEYFIK; VLEDSLK; VQQLIK; NTVIPTK; ITITNDQNR
(f)	Heat shock protein (hsp-1) <i>C. elegans</i>	VEIANDQGNRTTPSYVAFTDTER; VQVEYK; DAVVTPPAYFNDSQR; DAGAIAGLNVLRINEPTAAAIAYGLDKK; STAGDTHLGGEDFDNR; STMDPVEK; SQVHDIVLVGGSTR; LLSDLFSGK; QTIEDEKLLK
(g)	Disulphide isomerase <i>O. ostertagi</i>	ALAPEYEK; LDATVHGDVASK; DAASIVAWLK; TWIQANR; IMEFFGLKKDDLPVRLISLEEDMTKFKPDFAEINTENIVK; NFEQVAR; QLAPTWDK; VQSFTIKFFPAGSNKVIDYTGDR HKDDYGAFK; TVVIQYTVK; KPEDWDER
(h)	Calreticulin-like protein <i>H. contortus</i>	DTAAEFDELTPEK; YVNNDVDR; HLVGIADDNKGDK
(i)	Calumenin-like protein precursor <i>C. elegans</i>	VTEQVLSFVYK
(k)	Fructose-bisphosphate aldolase <i>E. multilocularis</i>	VIPALNGKLTGMAFR; LEKPASMDDIKK
(l)	Glyceraldehyde-3-phosphate dehydrogenase <i>T. circumcincta</i>	

and one protein of 40 kDa (Table 1, 1) was found with homology to glyceraldehyde-3-phosphate dehydrogenase of *Teladorsagia circumcincta* (two matching peptides of 27 amino acid length; gi|2275067). The other five proteins that were analysed by MS did not show any homologies to known proteins.

Comparing the protein bands of fraction 9 with other fractions with suppressive activity revealed that seven protein bands were shared and at the same time prominent in fraction 9. The molecular weights of these protein bands are: 85 kDa, 75 kDa, 70 kDa, 55 kDa, 50 kDa, 40 kDa and 38 kDa and include the two HSP proteins, calreticulin, the disulphide isomerase and the dehydrogenase identified by MS.

DISCUSSION

This study describes the use of a combination of methods for the identification of immunomodulating helminth proteins. We characterized a protein fraction of *H. polygyrus* extracts and ES-antigens that induced cellular hyporeactivity as well as down-regulation of NO production by macrophages. Components of that particular fraction were recognized by IgE and led to the degranulation of an IgE-sensitized basophil cell line. The reactive fraction comprised proteins of molecular sizes between 175 and 30 kDa. Mass spectrometry performed on 12 proteins revealed seven components with homologies to known proteins.

It is well established that helminth infections markedly modulate host immune responses, and both parasite-specific and non-specific immune suppression is well documented. Usually, during the acute phase of an infection antigen-specific T cell proliferation is stimulated, but with increasing exposure to parasite antigens the immune system becomes hyporesponsive (Figure 1) (1). The most frequently discussed hypotheses relate to modified T cell responses, regulatory T cells and alternatively activated macrophages. These mechanisms probably act in concert, which then causes a pronounced cellular hyporeactivity during the chronic phase of the infection.

As the knowledge on individual molecules of parasitic nematodes involved in the modulation of immune response is still very limited, we used *H. polygyrus* as a model system to characterize new immunomodulators. This parasite was shown to interfere with the expulsion of homologous and heterologous nematode infections and should therefore have a strong modulatory activity. We evaluated the modulatory capacity of protein fractions with three independent assays reflecting different immunologically relevant processes. First, we assessed whether the FPLC-separated proteins interfere with proliferative response of T cells. For this purpose we used T cells from OVA-TCR-transgenic mice,

which specifically respond to the OVA³²³⁻²²⁹ T cell epitope, and stimulated these cells with the entire OVA protein. This allowed us to identify proteins that interfere with antigen-specific T cell responses. Second, we tested the influence of nematode proteins on effector functions of macrophages, because the blocking of reactive nitrogen or oxygen intermediates is an important immune evasion mechanism. Third, we studied the capacity of the separated proteins to cross-link serum IgE antibodies of infected animals used for the sensitization of a basophil cell line, which then degranulates upon antigen contact. Interestingly, our data showed that one fraction of secreted worm proteins and of worm extracts interfered strongly with all three independent assays. This suggests that one molecule in this particular fraction is responsible for all detrimental effects, but by chance this one fraction could also contain several individual immunomodulators.

To identify individual immunomodulators we analysed peptide sequences of 12 bands of the separated extracts by high-pressure liquid chromatography coupled to mass spectrometry. This approach led to the identification of peptide sequences of seven proteins matching to sequences within the database. One candidate that particularly matches the observed effects is calreticulin (CalR). CalR is a multifunctional Ca²⁺-binding protein exhibiting multiple domains and binding sites (29). The *H. polygyrus* CalR protein band of about 55 kDa was found in all fractions with suppressive activities, but was most prominent in fraction 9 that exhibited the highest activity. The CalR-associated functions include calcium storage and signalling, cell adhesion, gene expression, and chaperoning of glycoproteins (30,31). CalR has been identified in several parasites, such as *Necator americanus* (32), *Onchocerca volvulus* (33), *Schistosoma mansoni* (34). CalRs of several parasites like e.g. *Trypanosoma cruzi* and *Haemonchus contortus* were shown to interfere with the activation of complement and were suggested to play a role in immune evasion by the parasites (30,35). Therefore, CalR of *H. polygyrus* could theoretically interfere with the cellular proliferation by regulating Ca²⁺-dependent processes operating in activation of T lymphocytes. Furthermore, CalR may interact with reactive nitrogen intermediates (30,36), which might explain its effect on the NO production of macrophages.

However, we cannot exclude the possibility that other proteins are responsible for the immunomodulatory effects found in this study. Two heat shock proteins (HSPs) identified by mass spectrometry might exert immunomodulatory properties, as HSPs are discussed as potent virulence factors in murine toxoplasmosis (37) and mycobacterial infection (38). Both *H. polygyrus* HSP proteins were prominent in fraction 9 and could also be detected in the other fractions with immunosuppressive activities.

Furthermore, a second Ca^{2+} -binding protein, calumenin, was identified in the reactive fraction. Calumenin is associated with the endoplasmic reticulum but has been suggested to be involved in pathological processes (39). In addition, a protein with homology to a disulphide isomerase of *Ostertagia ostertagi* was identified, which has been described to be immunogenic during infection (40,41). Two proteins of fraction 9 showed homologies to fructose-bisphosphate aldolase of *Onchocerca volvulus* (42) and a glyceraldehyde 3-phosphate dehydrogenase of *Schistosoma mansoni* (43), respectively, which were not previously mentioned in the context of immunomodulation.

It is not exactly clear how the IgE-binding capacity of immunomodulating protein(s) of fraction 9 could be related to the observed effect, but a coincidence of IgE binding and immunomodulation was also observed for cystatin, a well characterized filarial immunomodulator. In general it is believed that the induction of IgE in gastrointestinal nematodes participates in effector mechanisms that lead to nematode expulsion. However, it cannot be excluded that IgE production is an epiphenomenon of a process that leads to immunomodulation and further studies are necessary to clarify a possible relationship. In this context again calreticulin of the reactive fraction might be an interesting candidate, as that protein has been shown to induce IgE production and to contain IgE epitopes and lectin-binding domains (30,44).

It has been shown that *H. polygyrus* infection primes a Th2 response to an orally administered antigen (45) and that an adoptive transfer of T cells from *H. polygyrus*-infected mice into colitic recipients inhibited colitis (46) and allergic airway hyperresponsiveness (47). These data indicate that nematodes alter the host's immune response and may prevent exacerbated inflammation. Therefore, the precise mechanism of helminth-induced immune modulation as well as the identification of helminth molecules that are responsible for powerful parasite-induced immunosuppression might be extremely useful in the development of intervention strategies for harmful inflammatory reactions. Further studies that focus on the molecular cloning of relevant components of fraction 9, will allow us to characterize their immunomodulatory potential in detail.

ACKNOWLEDGEMENTS

The study was supported by grants of the Deutsche Forschungsgemeinschaft to SH and RL (SFB 618, SFB 650) and by the Polish State Committee for Scientific Research, KBN (3P04C 087 24) to MD. The excellent technical assistance of Bettina Sonnenburg and Marion Müller is acknowledged. The authors thank Johannes Gladitz for the professional statistical analyses of the data.

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