Non-specific binding of mouse IgG1 to *Heligmosomoides polygyrus*: parasite homogenate can affinity purify mouse monoclonal antibodies

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(*Received 29 April 1996; revised 17 July 1996; accepted 17 July 1996*)

SUMMARY

A characteristic feature of infections with the nematode parasite of mice *Heligmosomoides polygyrus*, is a marked IgG1 hypergammaglobulinaemia. A possible source for this immunoglobulin has recently been demonstrated, through evidence that *H. polygyrus* adult worm homogenate (AWH) can induce the *in vitro* production of non-specific IgG1 from mouse lymphocytes. To determine the interactions between this immunoglobulin and the parasite, the ability of IgG1 to bind to AWH of *H. polygyrus* was investigated. Protein (Western) blotting indicated that mouse monoclonal antibodies are able to bind non-specifically to selected parasite antigens. Furthermore, by binding *H. polygyrus* adult worm homogenate to cyanogen bromide (CNBr)-activated Sepharose CL-4B, an affinity column was prepared which could be used to efficiently purify mouse IgG1 monoclonal antibodies. These antibodies were eluted from the affinity column and still retained their original specificity. These results indicate that *H. polygyrus* not only induces the production of non-specific IgG1 by the host, it can also bind this immunoglobulin to its own specific proteins. Thus, it is possible that IgG1 produced during a primary infection with *H. polygyrus* may not entirely benefit the host.

Key words: *Heligmosomoides polygyrus*, nematode, affinity chromatography, IgG1.

INTRODUCTION

Heligmosomoides polygyrus is a nematode parasite of mice, in which the adult stages exist as a chronic infection in the duodenum of the host (Behnke & Robinson, 1985). In most strains of mice, a conspicuous feature of infections with *H. polygyrus* is a marked serum hypergammaglobulinaemia, which consists largely of immunoglobulins of the IgG1 subclass (Chapman *et al.* 1979*a*, *b*; Pritchard *et al.* 1983). This raised IgG1 has been demonstrated to be most prominent in immunized animals (Pritchard *et al.* 1983), and there are data to indicate that these antibodies have some host protective qualities (Behnke & Parish, 1979; Pritchard *et al.* 1983; Williams & Behnke, 1983). However, raised serum IgG1 is also a marked feature of a primary or chronic infection with *H. polygyrus* (Pritchard *et al.* 1983), and the function of the immunoglobulin produced under these circumstances has been disputed for a number of years.

Data have clearly shown that primary infection IgG1 has no ability to passively transfer immunity to naive animals (Williams & Behnke, 1983), although it has been postulated that these antibodies are probably host protective, but at low specific titre (Pritchard, Behnke & Williams, 1984). Conversely, some workers have proposed that rather than being host protective, IgG1 produced during a primary infection may actually block host protective immune responses (Chapman *et al.* 1979*a*, *b*). Nevertheless, there are results showing that serum IgG1 levels do correlate with a strain's ability to resist a primary infection with *H. polygyrus* (Wahid & Behnke, 1993), but the same group, when looking within strains, have found little correlation between IgG1 levels in individual mice (Wahid & Behnke, 1993).

We have previously shown that *H. polygyrus* adult worm homogenate (AWH) can induce the *in vitro* production of IgG1 from naive mouse splenocytes (Robinson & Gustad, 1996). Upon analysis, however, the immunoglobulin produced by this stimulation of mouse lymphocytes showed little or no specificity for the stimulating AWH (Robinson & Gustad, 1996), which leads to the question of a physiological role for this antibody. As Enriquez and coworkers have shown that *H. polygyrus* can bind to IgE, without utilizing the Fab portion of the immunoglobulin molecule (Enriquez, Boggavarupu & Bradley-Dunlop, 1992), the ability of mouse IgG1 to non-specifically bind to *H. polygyrus* was investigated. It is postulated that the parasite is able to bind the mouse IgG1 to its own proteins which could mask specific antigens and, furthermore, if the parasite can bind IgG1 non-specifically, this mech-

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anism could be utilized to extract IgG1 from complex protein mixtures.

MATERIALS AND METHODS

Mice

BALB/c mice were bred and maintained in the Department of Veterinary and Microbiological Sciences, NDSU. Mice in this colony have been regularly checked, and have been found to be negative for pinworm. Mice were a minimum of 2 months old at the time of experimentation.

Parasite

The production and maintenance of *Heligmosomoides polygyrus* was in outbred Swiss Webster mice, as described by Jenkins & Behnke (1977).

Antigen

Adult *H. polygyrus* worms were removed from the intestine of mice using a modified Baermann technique (Jenkins & Behnke, 1977). Worms were washed repeatedly in distilled water and aprotinin (Sigma) was added at 0·1 U/ml of worms, prior to homogenization using a ground-glass tissue homogenizer. The homogenate was centrifuged at 16600 ***g*** for 30 min at 4 °C and then the supernatant was removed and sterilized using a 0·2 μm filter. The protein concentration of the resultant *H. polygyrus* adult worm homogenate (AWH) was assayed using a BCA assay (Pierce), with BSA as the standard.

Monoclonal antibodies

Monoclonal antibodies (MoAb) 9A3, 13B3, 12A4, 5B2, HpD (all IgG1), 8B3, 6C2 (all IgM), to *H. polygyrus* AWH; MoAb EA3, FD3 (all IgG1) to mouse thymulin; MoAb 1A6 (IgM), to keyhole limpet haemocyanin (KLH), were produced in BALB/c mice at NDSU, according to accepted methodology (Goding, 1986). All other MoAb were produced from hybridomas purchased from American Type Tissue Culture (ATTC). The hybridomas 1B7·11 and 25-5-16S produce mouse IgG1 MoAb specific for 2,4,6 trinitrophenyl (TNP) and IgM specific for mouse Ia respectively. Hybridoma BP107·2 produces a mouse IgG3 MoAb specific for mouse Ia.

All MoAb were produced in cell culture and concentrated using 45% saturated ammonium sulfate (SAS), followed by dialysis. *H. polygyrus*-specific, hyperimmunized mouse serum (HIS) was produced in BALB/c that has been immunized during the production of the anti-*H. polygyrus* MoAb. Normal mouse serum (NMS) was pooled from several naive BALB/c mice, bred in the mouse colony at NDSU.

As thymulin is only 9 amino acids long (EAKSQGGSD), a thymulin–BSA conjugate was used as the antigenic stimulant and a thymulin–KLH conjugate was used to test for the specificity of the monoclonal antibodies produced. The thymulin-specific MoAb used in these studies do not bind to KLH in isolation (data not shown). The specificity of the hybridomas was tested by ELISA, with AWH or thymulin–KLH as the target antigen.

ELISA

For evaluation of specific immunoglobulin, plates were coated with 100 μl of coating buffer, containing either 10 μg/ml (AWH) or 2 μg/ml (thymulin) of protein, followed by blocking with 3% chicken egg albumin (CEA). SAS-precipitated immunoglobulin was then added. Bound antibody was detected using either alkaline phosphatase (AP) conjugated, rabbit anti-mouse IgG and IgM (Sigma) (Fig. 1), or biotinylated goat anti-mouse IgG, with a streptavidin-biotinylated AP complex (Fig. 5). The substrate, pNPP (paranitrophenyl phosphate, Sigma), was used according to the manufacturer's instructions. The reaction was quantified, by measuring the optical density at 405 nm, using a Biotek microplate reader.

SDS–PAGE

SDS–PAGE was carried out on 10% gels using Mini-PROTEAN II (Bio-Rad) electrophoresis equipment, according to the manufacturer's instructions. Low and high molecular weight markers were purchased from Bio-Rad. Mouse IgG1 (Sigma) and bovine γ-globulin (Accurate Chemical Company) were purchased as indicated. Gels were stained using a silver staining technique, according to published methodology (Merril *et al.* 1981).

Western (protein) blots

A total of 100 μg of *H. polygyrus* AWH was electrophoretically separated on a 10% SDS–PAGE gel, then blotted onto a PVDF (polyvinylidene fluoride) membrane, using Mini-PROTEAN II electrophoresis and blotting equipment (Bio-Rad), according to the manufacturer's instructions. The blot was blocked overnight with Tris-buffered saline (TBS), containing 3% BSA, with or without 1% NMS, then washed 3 times, for 10 min each wash, with TBS–0·2% Tween 20, prior to being transferred to a Mini-PROTEAN II Multi Screen apparatus (Bio-Rad) for probing. The blot was incubated with the appropriate monoclonal antibodies, diluted with TBS–1% BSA, for 1 h, then washed 3 times, followed by incubation for 1 h with AP-conjugated goat anti-mouse IgG and IgM polyclonal antibody (Pierce), diluted 1/5000 with TBS–1% BSA. This was followed by a further 3

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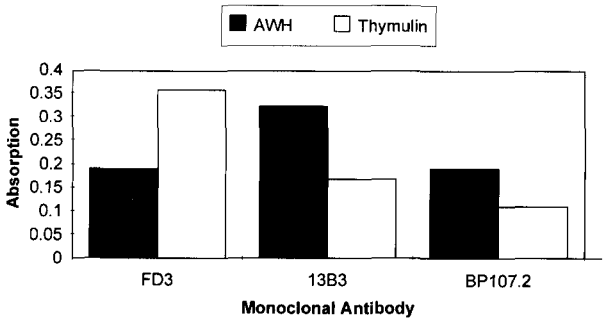

Fig. 1. ELISA to indicate the specificity of MoAb FD3, 13B3 and BP107.2 (all at 1:100) for thymulin and *Heligmosomoides polygyrus* AWH. Readings for PBS/Tween alone were 0·108 and 0·112 (AWH and thymulin, respectively).

washes. The blot was then removed to a plastic container and washed once in TBS for 10 min, followed by the addition of Bio-Rad Immuno-Blot colour development reagent, BCIP (5-bromo-4-chloro-3-indoyl phosphate) and NBT (nitroblue tetrazolium), in DMF (dimethylformamide) for 7 min. The reaction was stopped by washing with distilled water. All procedures were carried out at room temperature.

Affinity chromatography

A total of 50 mg of *H. polygyrus* adult worm homogenate (AWH) was incubated overnight with 3 g of pre-swelled CNBr-activated Sepharose CL-4B (Pharmacia) in coupling buffer (0·1 M $NaHCO_3$, 0·5 M NaCl, pH 8·3). The resulting mixture was then washed repeatedly with blocking buffer (0·2 M

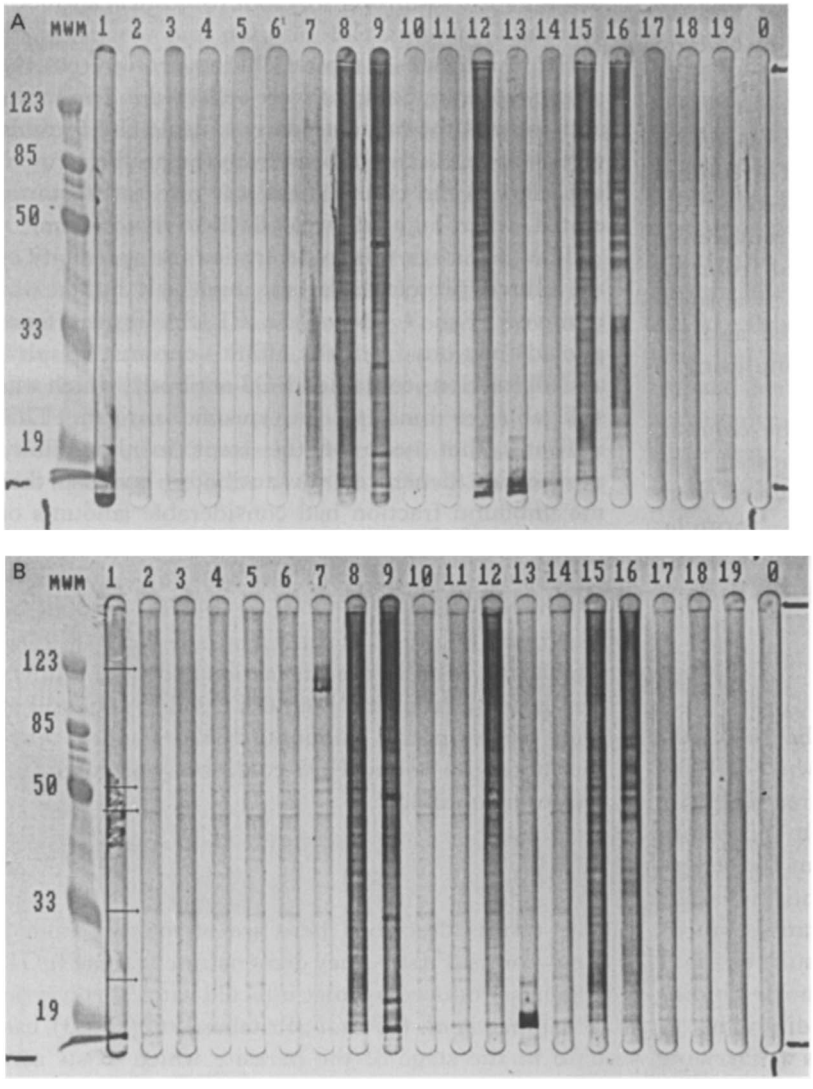

Fig. 2. Western (protein) blot to show the binding of MoAb to individual proteins of *Heligmosomoides polygyrus* AWH. The blocking buffers used were (A) TBS–3% BSA; (B) TBS–3% BSA–1% NMS. Antibodies and lanes were (1) 5B2 (100 μg/ml); (2) FD3 (10 μg/ml); (3) FD3 (100 μg/ml); (4) NMS (1:100); (5) buffer only; (6) hybridoma media only; (7) HpD (100 μg/ml); (8) 6C2 (100 μg/ml); (9) HIS (1:100); (10) BP107·2 (100 μg/ml); (11) 25–5-16S (100 μg/ml); (12) 9A3 (100 μg/ml); (13) 13B3 (100 μg/ml); (14) EA3 (100 μg/ml); (15) 8B3 (100 μg/ml); (16) 12A4 (100 μg/ml); (17) 1B7·11 (100 μg/ml); (18) 1A6 (100 μg/ml); (19) NMS (1:100); (20) blank.

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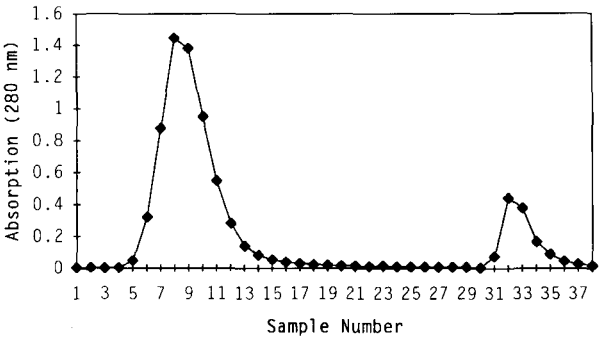

Fig. 3. Chromatogram showing the protein elution profile from the AWH affinity column. Samples 5–16 indicate unbound protein. Elution buffer was added at sample 26 and bound protein was eluted in samples 30–38.

glycine, pH 8·0), followed by alternative washes with acetate buffer (0·1 M sodium acetate, 0·5 M NaCl, pH 4·0) and coupling buffer, followed by a final wash with PBS. The AWH/Sepharose mixture was poured into a plastic liquid chromatography column and allowed to settle. A solution of 45% SAS precipitated MoAb, in loading buffer (20 mM MOPS, 20 mM NaCl, pH 7·2) was then allowed to pass through the column, under gravity. The column was washed with loading buffer, followed by the addition of elution buffer (4 M NaCl) to remove the purified MoAb. The antibodies removed from the column were quantified, approximately, by absorption at 260 nm and 280 nm, then diluted and the excess NaCl removed by centrifugation, using a Centriprep concentrator (Amicon).

To assay the antibody eluted from the column, protein fractions were collected as indicated, and desalted as required. Protein concentrations were adjusted to 0·5 mg/ml using the formula: $1{\cdot}55 \times A_{280} - 0{\cdot}76 \times A_{260}$ (= mg/ml), then their ability to bind to AWH was determined by ELISA, as detailed above.

RESULTS

Initial characterization of the MoAb to be used in the affinity column, was by determining whether FD3, specific for mouse thymulin, cross-reacted with AWH. Fig. 1 indicates that both MoAb 13B3 (anti-AWH) and FD3 bound specifically, but that cross-reactivity was low. MoAb BP107·2 (anti-mouse Ia) was also included in this assay as a negative control, and bound poorly to both antigens. Results of Fig. 1 are for 1/100 dilution of MoAb, but similar binding characteristics were observed at other dilutions.

The second stage of the analysis was to carry out protein blotting of AWH with various antibody preparations, both specific and non-specific for *H. polygyrus* AWH. The blots were carried out using 2 slightly differing methodologies, with the blocking solution consisting of TBS–BSA, but for one blot, NMS was included also. These 2 different methodologies produced quite different results, as indicated in Fig. 2. When the NMS mouse serum was omitted from the blocking buffer (Fig. 2A), the antibodies bound in discrete bands, as expected. However, with the addition of NMS to the blocking buffer (Fig. 2B), further bands appeared which were common to all antibody preparations tested.

These results indicated that a factor in NMS was binding to AWH proteins, which was then recognized by the goat anti-mouse antiserum of the second antibody. On the assumption that this factor might be mouse IgG1, we produced an affinity column using bound AWH, to determine if this could extract non-specific IgG1 from a semi-purified preparation of MoAb FD3. There were 2 factors which we wished to ascertain (i) whether IgG1 would bind to AWH with sufficient affinity to allow it to adhere to the column, and (ii) whether we could then extract the bound IgG1, in a way which would not denature the protein, or adversely affect antibody binding.

Fig. 3 indicates a typical elution profile from the affinity column. Samples were collected in 1 ml units and assayed for protein content, using the formula given above. As can be seen from the profile, protein did bind to the column and was also satisfactorily eluted, when high salt was added to the column.

The next stage was to determine the specificity of the eluted protein, after the high salt buffer was removed. Fig. 4 shows the ELISA results from pooled fractions from the affinity chromatography, and all fractions contained FD3 antibody, which was still able to bind to the specific antigen. This indicates that none of the steps in the affinity purification denatured the antibody, but also that the unbound fraction had considerable amounts of FD3 removed by the affinity purification process.

When the protein produced by affinity chromatography was analysed by SDS–PAGE, it could be seen that a large part of the bovine albumin (66 kDa) which had been in the unfractionated FD3 MoAb, was missing from the bound FD3, but was still at high levels in the unbound FD3 (Fig. 5). Furthermore, the bound FD3 could be seen to contain immunoglobulin.

DISCUSSION

The results described here are of interest from 2 view-points. Firstly, they demonstrate that the IgG1, which is produced by mice infected with *H. polygyrus* (Chapman *et al.* 1979*a*, *b*; Pritchard *et al.* 1983), can bind to the stage of the parasite which exists as a chronic infection within the host (Behnke & Robinson, 1985). We have not yet determined whether this property is true for other stages of the parasite's life-cycle, or for other preparations of adult worm proteins. As the specificity of the antibody is plainly not the reason for the binding,

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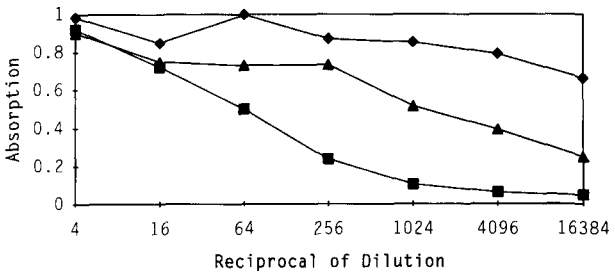

Fig. 4. Dilution curve indicating the specificity of MoAb FD3 for thymulin–KLH, as demonstrated by ELISA. (–◆–) Unf. (MoAb FD3 as applied to the column), (–■–) unbound protein, (–▲–) bound protein.

then one must assume that, as with IgE (Enriquez *et al.* 1992), the binding is not via the Fab region of the immunoglobulin molecule. These results would, therefore, indicate that the non-specific IgG1 produced by AWH (Robinson & Gustad, 1996), is not merely a by-product of stimulation of mouse lymphocytes, but does in fact interact with the stimulating organism. These data would probably add some credibility to the idea that the IgG1 produced during a primary infection with *H. polygyrus* does not act to benefit the host, but instead could act as an immunological blocking agent (Chapman *et al.* 1979*a*, *b*).

These results also indicate that the binding sites to which IgG1 adheres, bind BSA, CEA, bovine immmunoglobulins, and possibly other proteins. The data from the ELISA indicates that CEA can effectively block non-specific binding of the IgG1 to AWH, because this was the protein used in the blocking buffer. Similarly, the protein blots indicate that blocking the blots overnight with BSA, has the same effect, except that when NMS was added to the blocking buffer, mouse immunoglobulins were able to bind and additional bands resulted. There always exists the possibility of cross-reactivity between NMS and specific pathogens, due either to gut-living bacteria, or pinworm infections. However, our mice are frequently checked for pinworm infections and several MoAb, such as FD3, are able to bind AWH, indicating that antibody specificity is not a factor.

The ability of the affinity column to bind mouse IgG1 in the presence of other serum proteins indicates that, under the conditions utilized during these experiments, the affinity of AWH for mouse IgG1 is higher than for some other proteins. In regard to this latter point, we have also used the same column to determine if we could extract bovine immunoglobulins from the foetal calf serum contained in the hybridoma culture medium. Although we could not determine the specificity of these immunoglobulins, we could quantitatively measure them using an ELISA capture assay (Robinson & Gustad, 1996). We were successful in this (data not shown), showing that the affinity column also effectively binds bovine immunoglobulins. This probably accounts for the rather heterogeneous immunoglobulin light chain seen in the bound fraction in Fig. 5. Therefore, many serum proteins appear able to non-specifically bind to *H. polygyrus* AWH, although the evidence here would suggest that the binding affinities, under the conditions utilized here, vary considerably. This would be in accordance with data obtained using Protein A, as the affinity medium (Pritchard *et al.* 1984; Andrew & Titus, 1992).

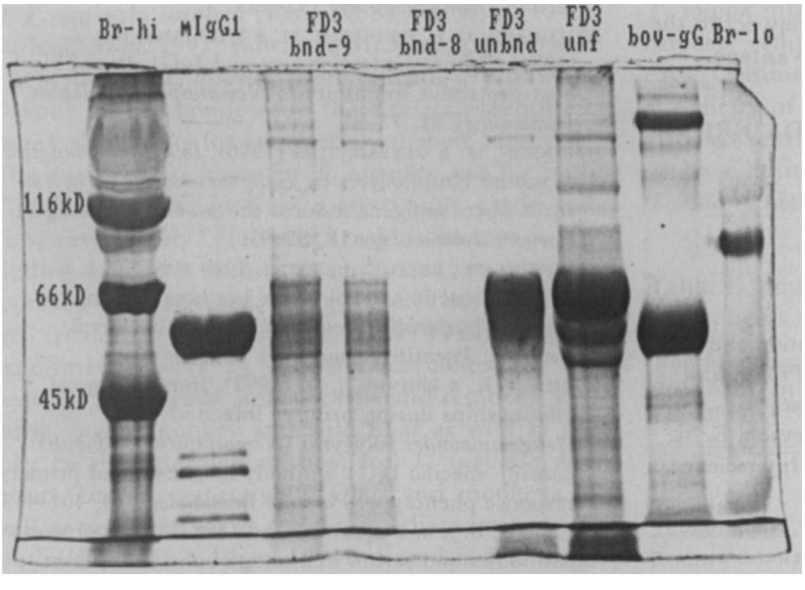

Fig. 5. SDS-PAGE gel of the protein fractions produced by affinity chromatography. Br-hi = high molecular weight marker; mIgG1 = commercial mouse IgG1; FD3 bnd-9, (4 and 2 μg, respectively) from the column used for Figs 3 and 4.; bnd-8 = bound FD3 from a small trial affinity column; FD3 (unbnd) = unbound FD3; FD3 (unf); unfractionated FD3; bov-gG = commercial bovine g-globulin; Br–lo = low molecular weight marker.

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The second point of interest is of a practical nature, because these data show that mouse IgG1 can be successfully isolated by affinity chromatography using AWH as the affinity medium. In this study we were not overly concerned with optimizing this procedure, but merely wished to demonstrate its effectiveness. Therefore, further work must be carried out to determine the ideal concentration of proteins and whether other buffer systems might be more suitable. However, we have used this method to successfully purify mouse IgG1 monoclonal antibodies. The methodology is a variation of established procedures, and effectively purifies IgG1 while still maintaining the viability of the immunoglobulin. This has some advantages over Protein A, for which buffers with extreme pH are often recommended, and also over Protein G, with which removal of the bound antibody can be difficult (Andrew & Titus, 1992).

Interestingly, this ability to bind proteins non-specifically could present problems during some assays. Although effective blocking when carrying out protein blotting and ELISA's should eliminate any false positives, the identification of antigenic proteins by immunoprecipitates could easily be affected. Therefore, results describing cross-reactivity between monoclonal antibodies and *H. polygyrus*, may need to be re-examined (Robinson *et al.* 1991).

In summary, these results demonstrate that serum proteins, including IgG1, are able to bind non-specifically to *H. polygyrus* AWH. This binding of IgG1 is competitive and reversible, and can be utilized during affinity chromatography in order to extract mouse IgG1. It is postulated that the parasite stimulates the host to produce non-specific IgG1, which can then bind to selected parasite proteins, and it is possible that this mechanism may act to the parasite's, rather than to the host's, advantage.

This work was supported in part by USDA/NRICGP Grant 94–37208–1036.

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