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Heligmosomoides polygyrus Elicits a Dominant Nonprotective Antibody Response Directed against Restricted Glycan and Peptide Epitopes

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Heligmosomoides polygyrus is a widely used gastrointestinal helminth model of long-term chronic infection in mice, which has not been well-characterized at the antigenic level. We now identify the major targets of the murine primary Ab response as a subset of the secreted products in *H. polygyrus* excretory–secretory (HES) Ag. An immunodominant epitope is an O-linked glycan (named glycan A) carried on three highly expressed HES glycoproteins (venom allergen *Ancylostoma*-secreted protein-like [VAL]-1, -2, and -5), which stimulates only IgM Abs, is exposed on the adult worm surface, and is poorly represented in somatic parasite extracts. A second carbohydrate epitope (glycan B), present on both a non-protein high molecular mass component and a 65-kDa molecule, is widely distributed in adult somatic tissues. Whereas the high molecular mass component and 65-kDa molecules bear phosphorylcholine, the glycan B epitope itself is not phosphorylcholine. Class-switched IgG1 Abs are found to glycan B, but the dominant primary IgG1 response is to the polypeptides of VAL proteins, including also VAL-3 and VAL-4. Secondary Ab responses include the same specificities while also recognizing VAL-7. Although vaccination with HES conferred complete protection against challenge *H. polygyrus* infection, mAbs raised against each of the glycan epitopes and against VAL-1, VAL-2, and VAL-4 proteins were unable to do so, even though these specificities (with the exception of VAL-2) are also secreted by tissue-phase L4 larvae. The primary immune response in susceptible mice is, therefore, dominated by nonprotective Abs against a small subset of antigenic epitopes, raising the possibility that these act as decoy specificities that generate ineffective humoral immunity. *The Journal of Immunology*, 2011, 187: 000–000.

Heligmosomoides polygyrus is a widely used experimental mouse model for the highly prevalent human and animal gastrointestinal helminth infections (1, 2). This system has provided major new findings in parasite immunology (3, 4), immune regulation (5, 6), nutrition (7), and ecology (8), and yet little information is available on the specific parasite Ags to which the host immune system is exposed. In this study, we set out to identify the molecular targets of murine humoral Abs, to define

individual *H. polygyrus* Ags, and to investigate the role of major Ab specificities in the host–parasite relationship.

Among the interesting facets of *H. polygyrus* is its ability to establish a chronic infection in most strains of laboratory mice, with the genetic background influencing the rate of expulsion rather than susceptibility per se (9–11). Genetically resistant mice mount a more rapid serum Ab response measured against adult worm somatic extract (12) or excretory–secretory (ES) Ags from cultured adult parasites (13, 14), and immunity to reinfection is compromised in B cell-deficient mice (4, 15–17). Early investigations had reported that passive transfer of serum from infected mice can confer a degree of immunity to *H. polygyrus* both in terms of worm number and in fecundity (18); this effect was associated with IgG1 isotype Abs (19, 20). More recently, IgG1 serum Abs have been demonstrated to reduce the fecundity and viability of adult worms and shown to require affinity maturation to confer any resistant effect (15).

As has been recently pointed out (21), in current nematode model systems, few serologically important Ags have yet been identified. Previous studies have relied on either crude whole-worm homogenates or collected secreted products as a more restricted but nevertheless complex antigenic set. Therefore, we decided to analyze the humoral Ab response to *H. polygyrus* in terms of specific Igs, to define the molecular targets of parasite-specific Abs, and to test whether these played any protective role against the infection in vivo. We tested Ab reactivities both to crude parasite antigenic extracts and also to preparations collected from in vitro culture of adult worms, termed “excretory–secretory” (ES) Ags, which are strongly implicated in immunomodulation of the host (6, 22). We report in this article that several major constituents are homologs of venom allergen *Ancylostoma*-

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Abbreviations used in this article: ASP, *Ancylostoma*-secreted protein; ES, excretory–secretory; ESI, electrospray ionization; HES, *Heligmosomoides polygyrus* adult excretory–secretory products; HES, *Heligmosomoides polygyrus* adult somatic extract; HM-65, high molecular mass 65 kDa antigenic complex; IP, immunoprecipitation; LC, liquid chromatography; MLN, mesenteric lymph node; MS/MS, tandem mass spectrometry; PC, phosphorylcholine; PNGase, peptide:N-glycanase; TFF, T follicular helper cell; TFMS, trifluoromethanesulfonic acid; VAL, venom allergen *Ancylostoma*-secreted protein-like.

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secreted protein (ASP)-like (VAL) Ags related to the vaccine candidates of human and canine hookworms (23, 24). However, the response to infection is dominated by anti-glycan specificities, and the murine Ab profile is highly restricted with respect to the range of Ags recognized.

Materials and Methods

Parasites, Ags, and mice

The original stock of *H. polygyrus bakeri* used in these studies was kindly supplied to us by Prof. J.M. Behnke (University of Nottingham, Nottingham, U.K.). Parasites, *H. polygyrus* ES (HES) Ag and adult worm somatic extract (HEX) were produced as described previously (6, 25, 26). Day 5 fourth-stage larvae were collected from the intestinal wall of infected mice and ES collected over a 3-d culture period in the same manner as adult HES. Female C57BL/6 and BALB/c mice (6–10 wk old) were bred in-house, and animal studies were performed under U.K. Home Office License. Mice were infected with 200 *H. polygyrus* L3 by oral gavage, and fecal egg counts and adult worm burdens were determined by standard procedures (2). For secondary infection, mice were treated orally with pyrantel embonate (27) in the form of 2.5 mg Strongid P paste in 0.2 ml water on days 28 and 29 postprimary infection. Drug-treated mice were rechallenged with 200 L3 by gavage 2 wk later. Where indicated, HES was heat denatured by incubating at 95°C for 20 min (6).

One-dimensional and two-dimensional gel electrophoresis and Western blotting

HES and HEX (1–10 µg) were separated, silver stained, or blotted as described previously (28). Blots were blocked in 2% BSA-TBS with 0.05% Tween 20 (TBST) for 2 h at room temperature, before being probed with

sera (1/500 dilution) or mAbs (2 µg/ml) at 4°C overnight. Following extensive washing in TBST, blots were incubated with HRP-conjugated secondary Abs (anti-mouse Ig 1/2000, Dako P0460, DakoCytomation; anti-mouse IgM 1/1000, Southern Biotech 1020-05, and anti-mouse IgG1, 1/2000 Southern Biotech 1070-05; Southern Biotechnology Associates) for 1 h at 37°C, washed in TBST, and then developed as described previously (28). Alternatively, IgA blots were incubated with biotinylated anti-mouse IgA (1/500, M31115; Invitrogen), followed by HRP-streptavidin (1/2000; Sigma-Aldrich), and developed as above. Mouse IgM mAb Bp-1 (29) was used for anti-phosphorylcholine (PC) blots at 1/1000 dilution and detected with anti-mouse Ig as above.

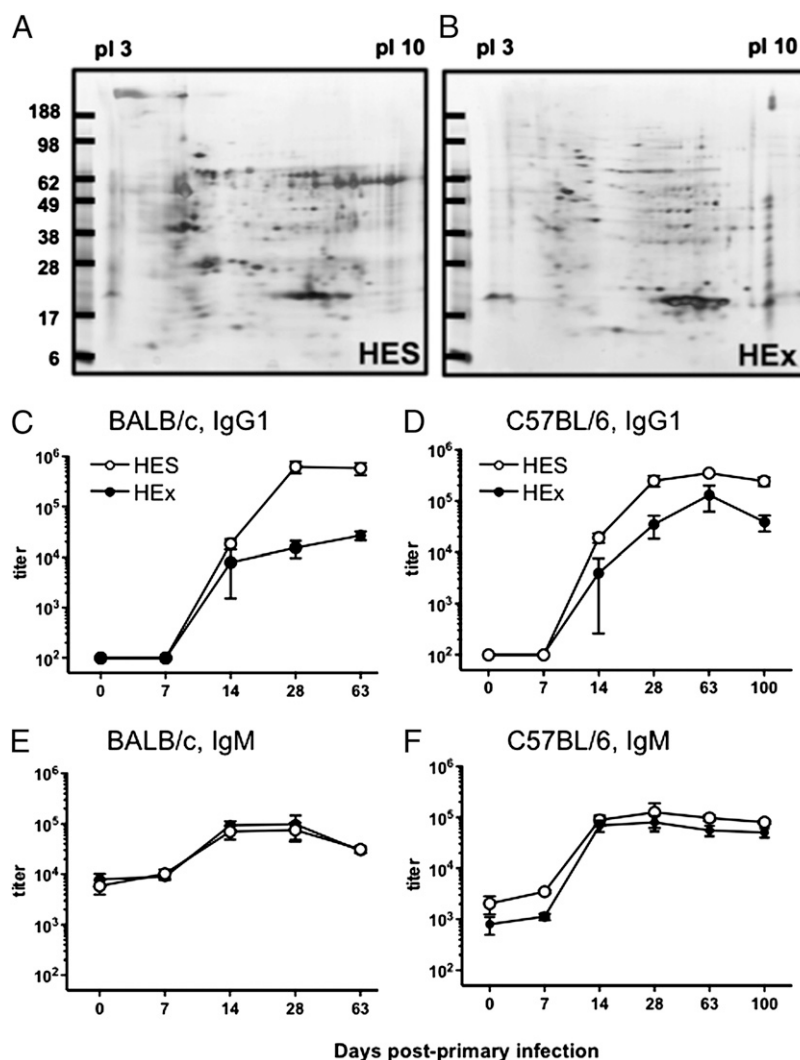
ELISA

HES and HEX (1 µg/ml) were coated on Immunoplates (Nunc) in 0.06 M carbonate buffer overnight (4°C), blocked with block solution (2% BSA-TBST) for 2 h (37°C), and then incubated with doubling dilutions of sera (in block solution) for 2 h (37°C). For comparison of HES and L4 ES, each were used to coat plates at a range of dilutions. Worm-specific Ab titers were detected using the secondary reagents described above and developed with ABTS (Insight Biotech). Titer was determined as the reciprocal dilution at which the sample dropped below background levels. For anti-PC ELISA, plates were coated with 1 µg/ml PC-conjugated BSA (30), serum was used at a 1/500 dilution and mAb at 5 µg/ml. Anti-*H. polygyrus* mAbs were used at 5 µg/ml for all ELISAs, unless stated. Goat anti-rat Ig (1/2000, Dako P0450; DakoCytomation) was used as a secondary for experiments with rat sera.

mAb and polyclonal Ab production

For mAbs, spleens and mesenteric lymph nodes (MLN) were recovered from C57BL/6 mice at day 28 postinfection and fused with SP2 cells. Fused cells were cultured for 12–14 d in HAT selection media (RPMI 1640

FIGURE 1. The predominant serological Ags of *H. polygyrus* are in HES rather than somatic extract. *A* and *B*, Two-dimensional silver-stained gels of 10 µg HES (*A*) and Hex (*B*). Molecular mass markers (in kDa) are indicated on the left. *C* and *D*, Anti-HES (open symbols) and HEX (solid symbols) IgG1 serum Ab titres from BALB/c (*C*) and C57BL/6 (*D*) mice at various time points following primary infection. *E* and *F*, Anti-HES and HEX IgM serum Ab titres in the same BALB/c (*E*) and C57BL/6 (*F*) mice. Each point represents the mean and SEM of data from five individual mice separately assayed. Data are representative of two independent experiments.



medium supplemented with 20% FCS [Hyclone], 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine [all Life Technologies], HAT [100 μ M hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine] and OPI [1 mM oxaloacetate, 0.45 mM pyruvate, and 0.2 U/ml insulin; all Sigma-Aldrich]. Plates were ELISA screened for the production of anti-HES Abs as above, and positive wells were cloned by two to three rounds of limiting dilution. Cells were then adapted into standard complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% FCS and the above concentrations of penicillin, streptomycin, and L-glutamine) and grown in bulk cultures in Vectra Cell Bioreactors (Bio-Vectra) to produce mAb. Ab isotypes were determined either with a mouse Ab isotype kit (Isostrip; Roche) or the anti-mouse Ig secondary Ab described above. Abs were purified using an AKTA prime fast protein liquid chromatography (LC) with a HiTrap protein G HP (IgG1 mAb) or HiTrap IgM purification (IgM and IgA) column, according to the manufacturer's instructions, and then dialyzed extensively into PBS. For the rat polyclonal Abs, rats were immunized with 25 μ g HES or recombinant *H. polygyrus* calreticulin (AM296015 (31), produced in *Escherichia coli*) in alum adjuvant i.p., then boosted with 10 μ g Ag on days 28 and 35, before serum collection on day 42. The mouse IgG1 myeloma MOPC 31C from American Type Culture Collection was used as an isotype control. A hybridoma-producing mouse anti-DNP IgM control was produced as above from mice immunized with 50 μ g DNP-conjugated keyhole limpet hemocyanin in alum adjuvant i.p. and then boosted on days 12 and 13 with 1 μ g DNP-keyhole limpet hemocyanin in PBS i.v. before spleen harvest on day 14. Screening was performed using DNP-OVA and anti-mouse IgM secondary Ab as above. Hybridomas were also produced from mice immunized with 25 μ g HES in IFA (Sigma-Aldrich) i.p. then boosted on days 48 and 49 with 1 μ g HES in PBS i.v. before spleen harvest and fusion on day 50.

Immunoprecipitation

HES was labeled with biotin (~40 μ g biotin reagent/100 μ g HES) using EZ-link Sulfo-NHS Biotinylation kit (Pierce) for 2 h on ice and then dialyzed overnight into PBS. Biotinylated HES was then precleared with protein G-agarose beads (16-266; Millipore) in the presence of MOPC 31C IgG1 isotype control for 30 min at room temperature. Unbound HES (2 μ g) was then incubated with 2 μ g various anti-HES IgG1 mAb, MOPC IgG1 control, or 5 μ l C57BL/6 day 28 primary or day 14 secondary infection sera, in nondenaturing immunoprecipitation (IP) buffer (20 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) for 2 h, and then with protein G-agarose beads overnight at 4°C with rotation. Beads were then washed 5 $\times$ 5 min in IP buffer, and bound proteins were eluted with 0.1 M glycine (pH 2.7). Eluted proteins were buffer exchanged into PBS (with MicroBio-Spin 6 chromatography columns; Bio-Rad), run on one-dimensional and two-dimensional gels and Western blotted as described above, and then probed with 1/2000 streptavidin-horseradish peroxidase (Sigma-Aldrich) before developing to allow visualization of biotinylated proteins.

Deglycosylation

For peptide:N-glycanase (PNGase) F treatment, HES or RNase B (Sigma-Aldrich) was denatured by heating to 95°C for 5 min in the presence of 0.1% SDS and 100 mM 2-ME, before the addition of 10 U PNGase F (Sigma-Aldrich) and 1% Triton X-100. Samples were incubated at 37°C for 3 h. For PNGase A treatment, HES was diluted in 100 mM ammonium bicarbonate (pH 8.5), heat denatured, and digested overnight with 1 μ g proteomics grade trypsin at 37°C (Sigma-Aldrich), after which trypsin was inactivated by boiling. Complete digestion of HES was confirmed by SDS-PAGE analysis (data not shown). Tryptic peptides were then lyophilized and resuspended in 100 mM sodium acetate (pH 5) and treated with 0.2 mU PNGase A (Roche) for 24 h at 37°C. For subsequent ELISA analysis, peptides were bound to plates in carbonate buffer at 1 μ g/ml and then tested for Ab binding as above. For PNGase F and A digestions, control proteins were treated in an identical manner but in the absence of enzyme. For chemical deglycosylation of HES with trifluoromethanesulfonic acid (TFMS), HES, or RNase B were dialyzed into 0.1 M ammonium acetate then lyophilized until completely dry. The pellet was resuspended in 10% anisole in TFMS (both Sigma-Aldrich) at 4°C, and the reaction was allowed to proceed for 2 h before neutralization with a 60% pyridine solution in a methanol-dry ice bath. The soluble fraction of HES was dialyzed into PBS, and the residual precipitate was solubilized in 1% SDS.

Affinity purification of VAL proteins

Twenty milligrams each of anti-VAL-1 (4-M15), 2 (4-S4), and 4 (2-11) mAbs were dialyzed into coupling buffer (0.1 M sodium bicarbonate and 0.5 M NaCl [pH 8.4]) and then reacted with swollen cyanogen bromide-

activated Sepharose beads (Sigma-Aldrich) overnight at 4°C with rotation. Unreacted groups were blocked with 0.2 M glycine (pH 8) for 2 h at room temperature, after which the beads were washed in five cycles of coupling

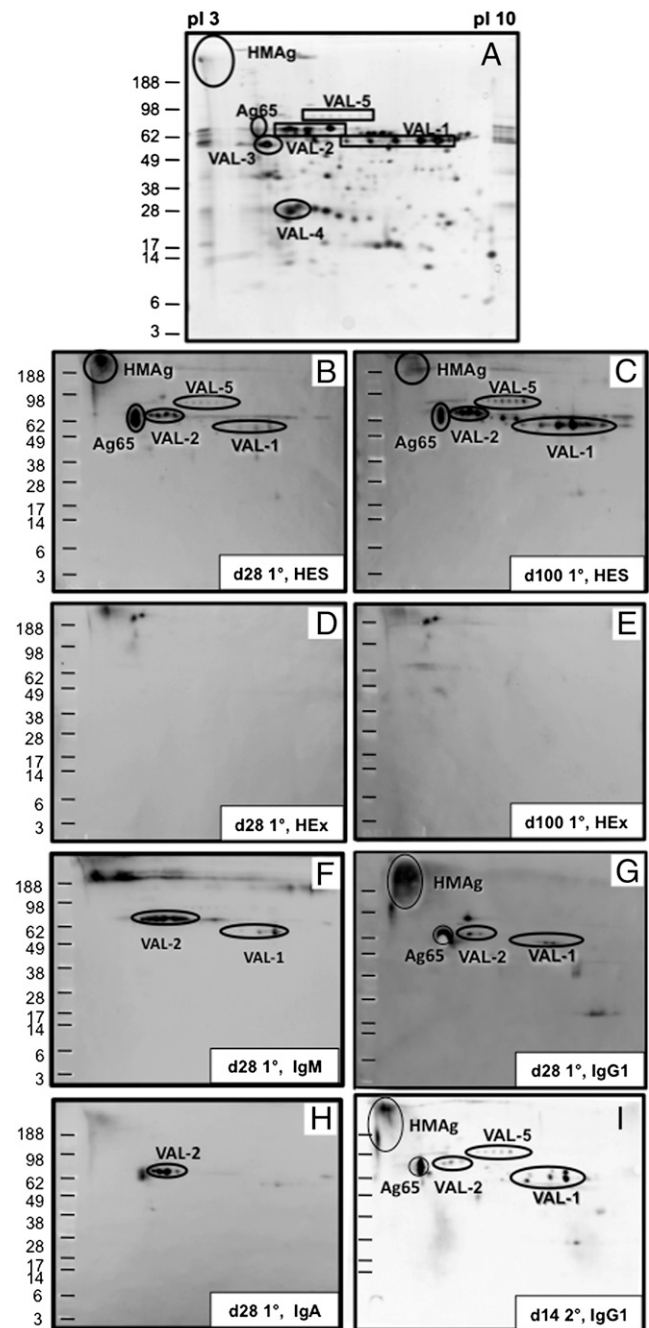


FIGURE 2. Polyclonal Abs recognize a restricted set of *H. polygyrus* Ags by Western blot. **A**, Silver-stained two-dimensional gel of HES indicating major components identified by mass spectrometry (34). HMAg and Ag65 indicate the position of Ags (subsequently termed HM-65; see Fig. 3D) that do not stain with silver. **B** and **C**, Two-dimensional Western blot of HES with sera from C57BL/6 mice taken at day 28 (**B**) and day 100 (**C**) following primary infection with *H. polygyrus*, developed with a polyvalent anti-Ig conjugate. **D** and **E**, As above, but with HEV. **F**, Two-dimensional Western blot of anti-HES IgM in sera from 28-d infected C57BL/6 mice. **G**, As above, for anti-HES IgG1. **H**, As above, for anti-HES IgA. **I**, Two-dimensional Western blot of anti-HES IgG1 in sera C57BL/6 mice collected 14 d following a secondary challenge infection. For each assay, sera were pooled from five mice. Molecular mass markers are indicated in kilodaltons, and results are representative of two or more experiments. Naive mouse sera showed no positive binding under the same conditions (data not shown).

buffer, followed by 0.1 M sodium acetate and 0.5 M NaCl (pH 4). For affinity purification, 10 µg HES was treated as before for IP, eluted proteins were run on one-dimensional SDS-PAGE, and bands of interest were excised for mass spectrometry analysis.

Sequence database and mass spectrometry

In studies to be published elsewhere, a database compiled of ~466,000 Roche 454 sequence reads from normalized and non-normalized adult *H. polygyrus* cDNA was assembled into ~20,000 predicted gene products (Y. Harcus, J.P. Hewitson, K.J. Filbey, J.R. Grainger, M. van Agtmaal, M. Thompson, N. Wrobel, S. Bridgett, M.L. Blaxter, and R.M. Maizels, manuscript in preparation). This database was used to match peptides identified by mass spectrometry of SDS-PAGE and two-dimensional gel-purified proteins. The individual protein genes described in this article (VAL-1, VAL-2, VAL-3, VAL-4, VAL-5, and VAL-7) were each amplified by PCR from *H. polygyrus* mRNA using gene-specific primers, and multiple independent clones were sequenced to verify the sequences predicted by the assembly algorithm. The full sequences for VAL-1 to VAL-5 and VAL-7 have been deposited with the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) under accession numbers JF914902, JF914906, JF914909, JF91410, JF914911, and JF914913, respectively. Immunoprecipitated HES proteins were prepared for mass spectrometry analysis as described previously (28). Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with an Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800–4000, and monoisotopic masses were obtained using a SNAP (sophisticated numerical annotation procedure) averaging algorithm. The 10 strongest peaks of interest, with a signal/noise ratio >30, were selected for tandem mass spectrometry (MS/MS) fragmentation in LIFT mode. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS. Identification of mAb 2-11 target VAL-4 required LC-electrospray ionization (ESI)-MS/MS using an Ultimate nanoLC system (Dionex) equipped with a PepMap C₁₈ trap (300 µm × 0.5 cm, Dionex) and an Onyx C₁₈ monolithic silica capillary column (100 µm × 15 cm; Phenomenex). Peptides were eluted over with a acetonitrile gradient (solvent A = 2% [v/v] acetonitrile, 0.1% [v/v] formic acid in H₂O; solvent B = acetonitrile, 0.1% [v/v] formic acid; gradient conditions consisted of 3 min solvent A), and then, a linear 0–50% gradient of solvent B over 20 min was applied, followed by a 50-min wash at 95% solvent B. The flow rate was 1.2 µl/min, and the column temperature was 60°C. The nanoLC was interfaced with a high capacity trap ultra ETD II ion-trap LC-MS/MS system (Bruker Daltonics) with an online nanoESI source. Positive ESI-MS and MS/MS were acquired using AutoMSn mode, over the 300- to 1800- m/z range. Instrument control, data acquisition, and processing were performed using Compass 1.3 SP1 software (Esquire control, Hystar, and DataAnalysis; Bruker Daltonics). MS/MS data were submitted to database searching against an in-house database containing the predicted gene products using the Mascot program (Matrix Science, version 2.1), through the Bruker ProteinScape interface (version 2.1). Database searching was run with a peptide tolerance of 250 ppm and MS/MS tolerance of 0.5 Da. Peptide matches with expect values <0.05 at a Mowse significance threshold of $p < 0.05$ were considered significant.

Immunofluorescence

For sections, adult *H. polygyrus* worms were snap-frozen on dry ice in Cryo-M-Bed mountant (Bright Instruments). Cryostat sections (5 µm; Leica) were cut onto Polysine slides (VWR), dried, and then fixed in 100%

acetone for 10 min. Sections were washed twice with PBS for 10 min and then incubated with the various mAb (50 µg/ml in PBS containing 1% FCS) for 2 h at room temperature, washed twice in PBS as before, and then incubated with secondary anti-mouse Ig tetramethylrhodamine isothiocyanate (1/100 in PBS) for 1 h at room temperature. Following extensive washing, sections were mounted in anti-fade Vectashield mountant (Vector Laboratories). Staining was visualized with an Olympus fluorescent microscope. Non-fixed intact worms were stained on ice in round-bottom 96-well plates and then treated as above.

Radiolabeling of adult worm surface

Adult *H. polygyrus* were surface radiolabeled essentially as described in earlier publications (32, 33). Eppendorf tubes (1.5 ml) were coated with 200 µl of a 1 mg/ml solution of Iodination reagent (Pierce) in chloroform. Once dried, the tubes were washed several times with PBS, before transfer of ~500 adult worms and 500 µCi [¹²⁵I] (PerkinElmer) on ice. The sample was incubated with frequent agitation for 10 min and then quenched by the addition of a saturated solution of *L*-tyrosine (Sigma-Aldrich). Radio-labeled parasite surface material was then produced as described above as with HEx, except parasites were homogenized in 1.5% n-octyl glucoside detergent and 1% protease inhibitor mixture (P8340; Sigma-Aldrich). Immunoprecipitates were performed as above (anti-HM-65 mAb 9.1.3 or rat anti-HES polyclonal Ab) or with anti-mouse IgM-agarose (A4540; Sigma-Aldrich). Autoradiographs were carried out on dried gels as described previously (32, 33).

Vaccination and passive immunization

C57BL/6 females were immunized with 25 µg HES in alum adjuvant i.p., then boosted on days 28 and 35 with 5 µg HES–alum i.p. Mice were challenged with 200 *H. polygyrus* L3 larvae, fecal egg counts were determined at days 14 and 28 postinfection, and adult worms counted at day 28. For passive immunization, C57BL/6 females were treated on day –1 and then every 2–3 d postinfection (with 200 *H. polygyrus* L3 larvae) with either 0.2 or 1 mg mAb i.p. (for IgG1 mAbs) or i.v. (for IgM mAbs) as detailed in the figure legend. Eggs and worm numbers were determined as above.

Results

Ab responses are predominantly directed at secreted, rather than somatic, parasite Ags

As a first step in defining immunogenic products of adult *H. polygyrus*, we compared a conventional antigenic preparation comprising a soluble whole parasite extract (HEx), with products released by live parasites maintained in serum-free tissue culture medium (HES). The overall protein compositions of HES and HEx are very different, as shown in Fig. 1A and 1B, respectively, and confirmed by recent proteomic analysis (34).

As IgG1 Abs predominate and have a protective role in both primary and secondary *H. polygyrus* infection (15, 19, 20), we then compared IgG1 responses to HES and HEx following a primary infection. Ag-specific IgG1 responses to HES were detected by ELISA in both BALB/c and C57BL/6 mice by day 14 post-infection and by day 28 reached a high titer, which was main-

Table I. mAbs to HES Ags

Ag, Specificity	Clone	Source	Isotype
Glycan A (VAL-1-2-5)	13.1, 2-2, 2-12, 2-13, 2-62, 3-8, 3-11, 3-28, 3-29, 3-40, 3-42, 3-55	d28 SPL	IgM
Glycan B (HM-65)	14.3	d28 SPL	IgA
	3-31	d28 SPL	IgM
	4-M9	d28 MLN	IgM
	4-M7, 4-M17	d28 MLN	IgG1
VAL-1	9.1.3	HES/IFA	IgG1
	2-6, 3-6, 3-10, 3-38, 3-39	d28 SPL	IgG1
	4-M4, 4-M15, 4-M20, 4-M23, 4-M25	d28 MLN	IgG1
VAL-2	4-S4	d28 SPL	IgG1
VAL-4	2-11	d28 SPL	IgG1

d28 SPL, day 28 spleen.

tained for at least 63 (BALB/c; Fig. 1C) and 100 d (C57BL/6; Fig. 1D). Moreover, IgG1 titers to HES were up to 20-fold higher than those to HEx and showed less variation between individual animals. We therefore focused in the majority of subsequent Ab investigations on HES.

The anti-parasite IgM response differed from the IgG1 response in several regards (Fig. 1E, 1F). Significant background levels of both anti-HES and anti-HEx IgM Abs were noted in naive mice, and following infection, specific IgM titers reached a relatively early plateau (day 14). Furthermore, reactivity to HES and HEx was equivalent at all time points. We were unable to detect the target of (presumably natural) IgM Abs present in naive mice

using both Western blot and mAb techniques, perhaps because of their relatively weak affinity (data not shown).

Ag specificity of polyclonal Ab responses

To identify individual antigenic targets of the Ab responses, we adopted both polyclonal and monoclonal strategies. First, we used two-dimensional SDS-PAGE separation of HES Ags, by which ~100 distinct protein spots are observed (Figs. 1A, 2A); the identity of most major proteins has been determined by mass spectrometry (34). Despite the abundance of potential Ags, however, the two-dimensional Western blot profile of polyclonal sera from C57BL/6 mice with 28-d primary infection is much more

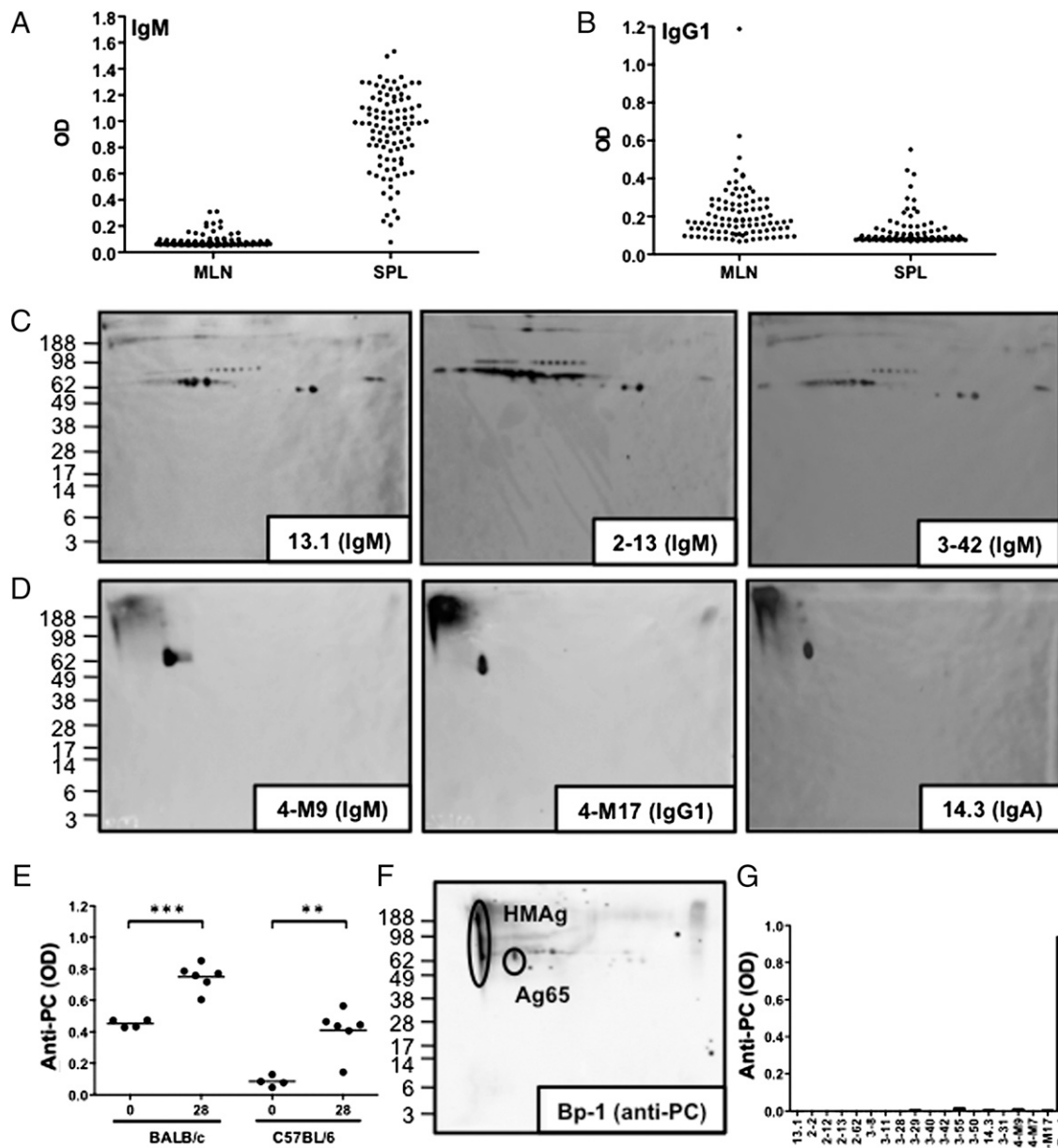


FIGURE 3. HES-specific mAbs from mice infected with *H. polygyrus*. *A* and *B*, Frequency of HES-specific hybridoma cells producing IgM (*A*) or IgG1 (*B*) from the MLNs and spleens of infected C57BL/6 mice at day 28 postinfection, as determined by ELISA. Each dot represents a single well of a 96-well plate. *C*, Representative two-dimensional Western blots of three different IgM anti-HES mAbs derived from infected mice, which bind to glycan A. *D*, Representative two-dimensional Western blots of anti-HES IgM, IgG1, and IgA mAbs recognizing glycan B on the HM-65 Ags. *E*, Anti-PC Abs measured by ELISA against PC-BSA in naive and 28-d infected sera from BALB/c and C57BL/6 mice. *F*, Two-dimensional Western blot of HES probed with anti-PC mAb Bp-1. The positions of the HM-65 Ags are circled. *G*, Reactivity to PC measured by ELISA on BSA-PC-coated plates; data from a panel of anti-HES mAbs are shown, together with Bp-1-positive control. Molecular mass markers are indicated in kilodaltons. ** $p < 0.01$, *** $p < 0.001$.

restricted (Fig. 2B). These were identified by proteomics as members of the venom allergen/VAL family of proteins (35, 36), specifically VAL-1, VAL-2, and VAL-5 (Fig. 2A) (34). Additional reactivity was noted to both a high molecular mass component and a smaller 65-kDa Ag, which do not appreciably silver stain and did not give measurable peptides for proteomic analysis; we term this Ag combination HM-65 (see below).

As reactivity increases over a long time frame, and as the C57BL/6 mouse is considered to be a slow responder to infection (37, 38), we also examined the serological response at 100 d postinfection (Fig. 2C); at this time point, responses to all the Ags previously noted were substantially stronger, but there remained a restricted repertoire of target Ags.

Consistent with ELISA results indicating that anti-HEX somatic extract responses were relatively weak, the same sera showed only slight reactivity by Western blot (Fig. 2D, 2E). The primary anti-VAL Ab response to HES was predominantly of the IgM isotype, although a smaller amount of anti-VAL-2 IgA was also noted (Fig. 2F, 2H). In contrast, anti-HES IgG1 response was detected against HM-65 (Fig. 2G). Polyclonal IgE did not provide a measurable signal by Western blot (data not shown).

We also examined the secondary response mounted by genetically susceptible mice cleared of infection by chemotherapy; in these mice, protective immunity is stimulated against challenge infection (9, 39). Importantly, immunity to challenge infection is ablated in B cell- or Ab-deficient mice (4, 15, 16, 40). By Western blot analysis, secondary IgG1 Abs showed a similar profile to samples from primary infection, albeit with a 10- to 30-fold higher titer (data not shown) and correspondingly stronger binding patterns (Fig. 2I). In contrast, secondary IgM responses were similar to primary Abs with respect both the titer and specificity profile (data not shown).

mAb specificities

We next generated a panel of monoclonal Abs to dissect the antigenic response in fine detail, using spleens and draining MLN

from infected mice at day 28 (Table I). Despite taking cells at this relatively late time point in the primary response, a substantial proportion of monoclonals represented Abs that had not undergone class switch from the IgM isotype, particularly when splenocytes rather than MLN cells were used (Fig. 3A). The great majority (12 of 14) of these IgM mAbs displayed a similar Western blot profile against HES (Fig. 3C) and bound to proteins previously identified as VAL-1, VAL-2, and VAL-5 by proteomic analysis. In fact, this indicates that the observed specificity of polyclonal serum (Fig. 2B, 2C) can be largely replicated by a single (monoclonal) Ab specificity and that an immunodominant epitope is shared by at least three members of the VAL family.

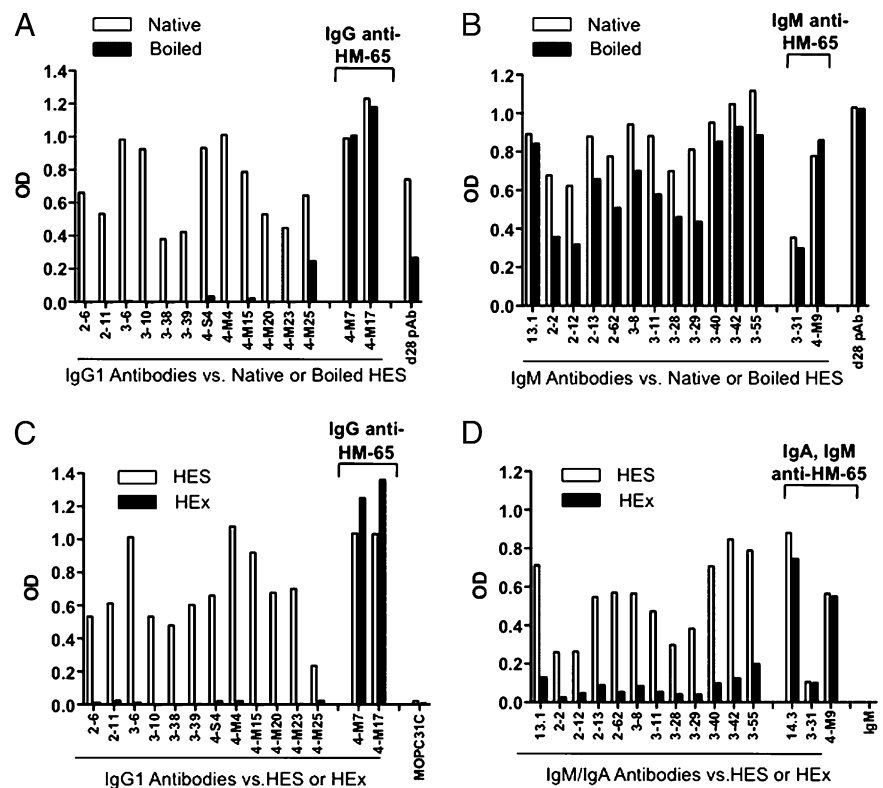
A different pattern was shared by a smaller number of Abs, including IgM, IgG1, and IgA isotypes, which bound to HM-65 (Fig. 3D). Notably, these components do not stain with silver and do not give measurable peptides for proteomic analysis, mimicking the pattern seen with polyclonal IgG1 from primary infections (Fig. 2G).

Because of the prominence of PC in many helminth products, including high molecular mass species with nonproteinaceous composition (30, 41), we tested for PC reactivity in infection sera and for PC epitopes in HES. Serum Abs from *H. polygyrus*-infected mice showed a modest degree of anti-PC binding (Fig. 3E), as has been reported previously (42). When two-dimensional Western blots were probed with monoclonal anti-PC Ab, the major positive reaction was with HM-65 (Fig. 3F). Despite this, none of the anti-HES monoclonals bound directly to PC (Fig. 3G), indicating that they target a non-PC specificity.

IgG Abs recognize heat-labile epitopes of secreted VAL Ags

The majority of IgG1 mAbs raised from infected mice (12 of 14) failed to react with HES by Western blot, despite their strong reactivity to native HES by ELISA (Fig. 4A and data not shown). We interpreted this to mean that the IgG1 response is predominantly directed against conformational protein epitopes, which

FIGURE 4. Most HES-specific IgG1 Abs recognize heat-labile epitopes absent from parasite extract. **A**, ELISA reactivity of anti-HES IgG1 mAbs to native (□) and boiled (■) HES. Polyclonal sera from 28-d infected C57BL/6 mice were included as a positive control. **B**, As above, for IgM mAbs. **C**, ELISA reactivity of anti-HES IgG1 mAbs to HES (□) and HEX (■). MOPC 31C myeloma IgG1 was included as a negative control. **D**, ELISA reactivity of anti-HES IgM and IgA mAbs to HES (□) and HEX (■). Anti-DNP IgM mAb was included as a negative control.



are destroyed following detergent denaturation during SDS-PAGE. In support of this, although IgM monoclonals are equally reactive to native or heat-denatured HES (Fig. 4B), heat denaturation of HES ablated all IgG1 mAb ELISA reactivity, with the exception of the two anti-HM-65 IgG1 Abs (Fig. 4A). Furthermore, although most IgG1 mAbs (Fig. 4C) and IgM mAbs (Fig. 4D) show little reactivity to somatic extract (HEX), all Abs specific for HM-65 show equally strong binding to HES and HEX.

To identify the heat-labile determinant(s) recognized by the IgG1 mAbs, an immunoprecipitation strategy was used using biotin-labeled nondenatured HES and protein G beads. The majority of these IgG1 monoclonals (10 of 14) were specific for a band that migrates in the position of VAL-1, as represented by 4-M15 (Fig. 5A). Two additional mAbs immunoprecipitated Ags comigrating with VAL-2 (4-S4) and VAL-4 (2-11).

Two-dimensional analysis of Ags immunoprecipitated by day 28 polyclonal infection serum confirmed that the dominant target of

primary IgG Ab in C57BL/6 mice was VAL-1 (Fig. 5D), as well as a spot that comigrates with another VAL protein abundant in HES, VAL-3 (Fig. 2A). Moreover, secondary IgG immunoprecipitated two further homologs, VAL-4 and VAL-7 (Fig. 5E). Comparison of profiles from Western blot (Fig. 2G, 2I) and immunoprecipitation (Fig. 5D, 5E) indicates that Western blotting gives an incomplete picture of IgG1 Ab specificity, as a result of the predominantly conformationally dependent VAL epitopes, whereas immunoprecipitation of biotinylated Ags omits the HM-65 group of Ags with low protein content.

To formally identify the immunoprecipitated proteins, samples bound by each Ab were eluted from one-dimensional gel bands (Fig. 5F) and subjected to mass spectrometry, matching the respective protein sequences (Fig. 5G). These assays also indicate that VAL-1, VAL-2, and VAL-4 do not interact with other proteins present in HES, given the absence of any coimmunoprecipitated components.

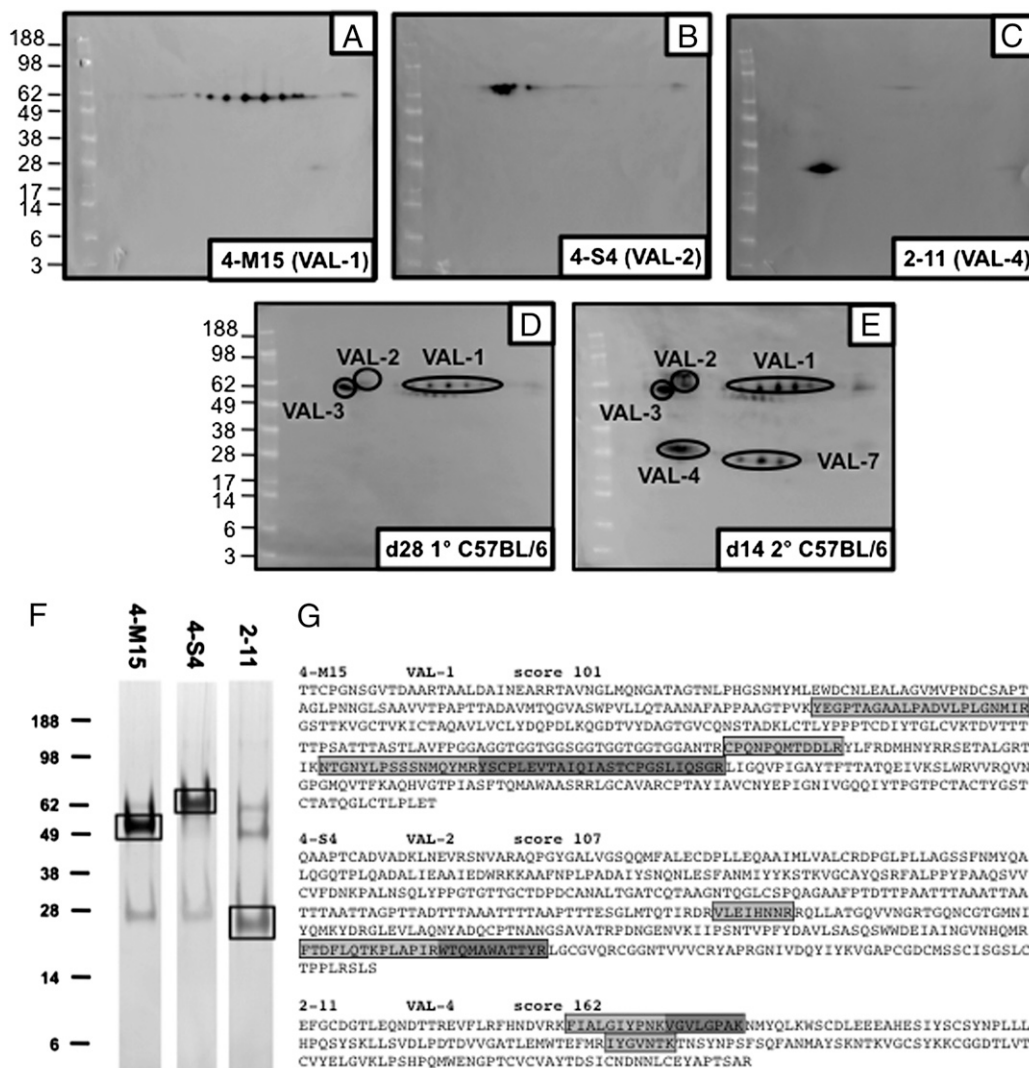


FIGURE 5. Conformation-dependent IgG1 Abs primarily target VAL Ags. *A–E*, Immunoprecipitation of biotin-labeled HES by the indicated mAb or polyclonal infection sera, separated by two-dimensional SDS-PAGE, and visualized by Western blotting with streptavidin–HRP. *A*, Anti-VAL-1 mAb 4-M15. *B*, Anti-VAL-2 mAb 4-S4. *C*, Anti-VAL-4 mAb 2-11. *D*, Primary day 28 C57BL/6 polyclonal infection serum. VAL-1, VAL-2, and VAL-3 are indicated. *E*, Secondary day 14 C57BL/6 polyclonal infection serum. Positions of VAL-4 and VAL-7 are indicated, as well as VAL-1, VAL-2, and VAL-3 as above. *F*, One-dimensional SDS-PAGE of immunoprecipitated (unlabeled) HES proteins using bead-conjugated mAb as indicated; boxes indicate segments eluted for mass spectrometric analysis. Additional bands likely reflect H and L chains of mAb leaching from beads. Molecular mass markers are indicated in kilodaltons. *G*, Peptides of VAL-1, VAL-2, and VAL-4 matched by mass spectrometry indicated by boxes. Mascot scores are also shown.

IgM Abs recognize a common O-linked glycan epitope on VAL glycoproteins

Despite being bound by several IgM monoclonals (Fig. 3C), the target Ags VAL-1, VAL-2, and VAL-5 show only limited amino acid homology (14.9% identity, 23.5% similarity; Supplemental Fig. 1), and reactivity is heat stable (Fig. 4B). We therefore evaluated the possibility of a carbohydrate nature of the target epitope(s). We also noted that the Ags' predicted molecular masses, based on primary amino acid sequences, were 10–39 kDa lower than their observed gel migration, yet each contained only a single potential N-linked glycosylation site (Fig. 6A). However, they encoded abundant serine and threonine residues in a central domain, which were predicted to be O-glycosylated by the NetOGlyc 3.1 program (Fig. 6A, Supplemental Fig. 2) (43). This contrasts with another secreted VAL protein of similar abundance, VAL-3 (Fig. 2A), which does not appear to be recognized by IgM Abs, lacks predicted O-glycosylation sites (Supplemental Fig. 2), and migrates on two-dimensional gels in a manner consistent with its predicted molecular mass (Figs. 3A, 6A).

To first confirm that glycan A is carried on VAL-1 and VAL-2, we showed that the anti-glycan A mAb 13.1 bound to VAL-1 and VAL-2 on Western blots following their affinity purification using IgG1 mAbs to the conformational epitopes of the VAL Ags (Fig. 6B and data not shown). Comparison with the profile of immunoprecipitated VAL-1 Ag (Fig. 5A) indicates that only three of the six VAL-1 spots react with the anti-glycan A mAb, as is also evident from Fig. 3B.

To then investigate the potential role of antigenic carbohydrates, we used both enzymatic and chemical deglycosylation strategies. When HES was pretreated with PNGase F to remove N-linked carbohydrates, small mobility shifts were evident in silver-stained gels (Fig. 6C) and Western blots (Fig. 6D), indicating the removal of N-glycans, yet polyclonal sera and IgM mAb reactivity remained intact (Fig. 6D, 6E). Similarly, the dominant Ag is unlikely to be an PNGase F-resistant N-glycan with a core α 1,3-fucose, because PNGase A treatment of tryptic HES peptides failed to ablate either polyclonal or mAb binding (Supplemental Fig. 3). Furthermore, mass spectrometric analysis identified a number of peptides containing unconjugated N-glycosylation sites (NxS/T), calling into question whether these are used in the VAL proteins of this species (data not shown).

To assess the role of O-glycans, HES was treated with TFMS, which removes both N- and O-glycans. Such chemical deglycosylation resulted in mobility shifts of silver-stained HES bands, indicative of glycan removal, comparable to that seen with a control glycoprotein, RNase B (Fig. 7A). Importantly, TFMS treatment ablated both anti-VAL IgM mAb and polyclonal infection sera recognition of HES (Fig. 7B, 7C), whereas TFMS-treated HES retained reactivity with an anti-protein Ab, raised against recombinant *H. polygyrus* calreticulin, as well as polyclonal serum from rats immunized with HES (Fig. 7D). These data therefore indicate that the dominant and persistent IgM Ab response is to an O-linked glycan shared by several polypeptide secreted Ags of *H. polygyrus*. The status of the HM-65 Ag is less clear; although PNGase F treatment ablates binding of anti-glycan B 14.3 mAb to the 65-kDa component but not the high molecular mass complex, the reverse is true for TFMS (Figs. 6D, 7B). Because the 65-kDa Ag is less abundant, the overall reactivity of 14.3 to TFMS-treated HES is reduced ~10-fold but not abolished (Fig. 7E). Thus, a similar or identical epitope is conjugated through different linkages to different carrier macromolecules. It was also noted that TFMS does not remove the PC moiety from HM-65 (data not shown), indicating that a different linkage is used in *H. polygyrus* from that described through N-linked glycans for *Acanthocheilonema viteae* (44).

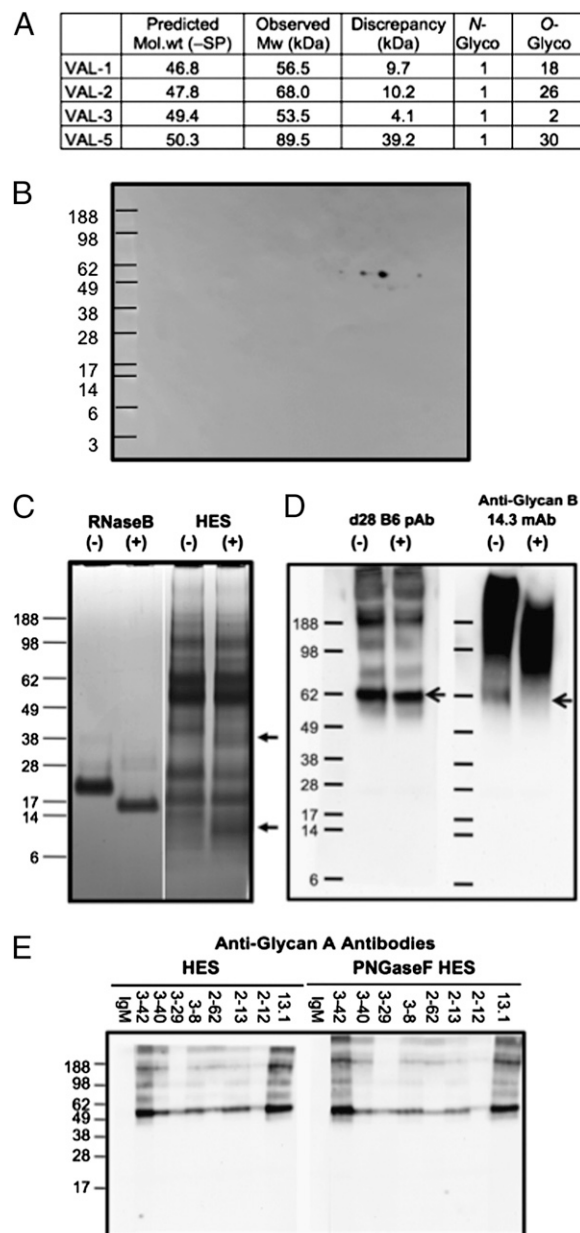


FIGURE 6. The immunodominant IgM target is not an N-linked glycan. A, Molecular mass discrepancies between the predicted molecular mass of indicated VAL proteins, based on amino acid sequence and observed migration on two-dimensional gels. Mature molecular mass is that predicted without the signal peptide (SP) sequence, predicted N-glycosylation sites are defined as N(X)S/T, and O-glycosylation sites were predicted using NetOGlyc version 3.1 (43). The proportion of predicted sites used is not known. B, Western blot of anti-VAL-1 mAb (4-M15)-precipitated Ag separated on two-dimensional SDS-PAGE and probed with anti-glycan A mAb (13.1). C, Silver-stained one-dimensional SDS-PAGE of PNGase F-treated (+) or mock-treated (-) RNase B control glycoprotein and HES. D, One-dimensional Western blot of HES with or without PNGase F treatment using day 28 C57BL/6 polyclonal sera and anti-glycan B (HM-65) mAb 14.3. Band shifts associated with enzymatic removal of N-glycans are arrowed. E, One-dimensional Western blots of HES with or without PNGase F treatment using anti-glycan A mAbs. IgM denotes an anti-DNP IgM mAb negative control. Molecular mass markers are indicated in kilodaltons

Glycans A and B as well as VAL-1 and VAL-4, but not VAL-2, are expressed by tissue-phase larvae

Upon infection of mice, *H. polygyrus* larvae first invade the submucosal tissue of the intestinal tract, molt twice (from L3 to L4

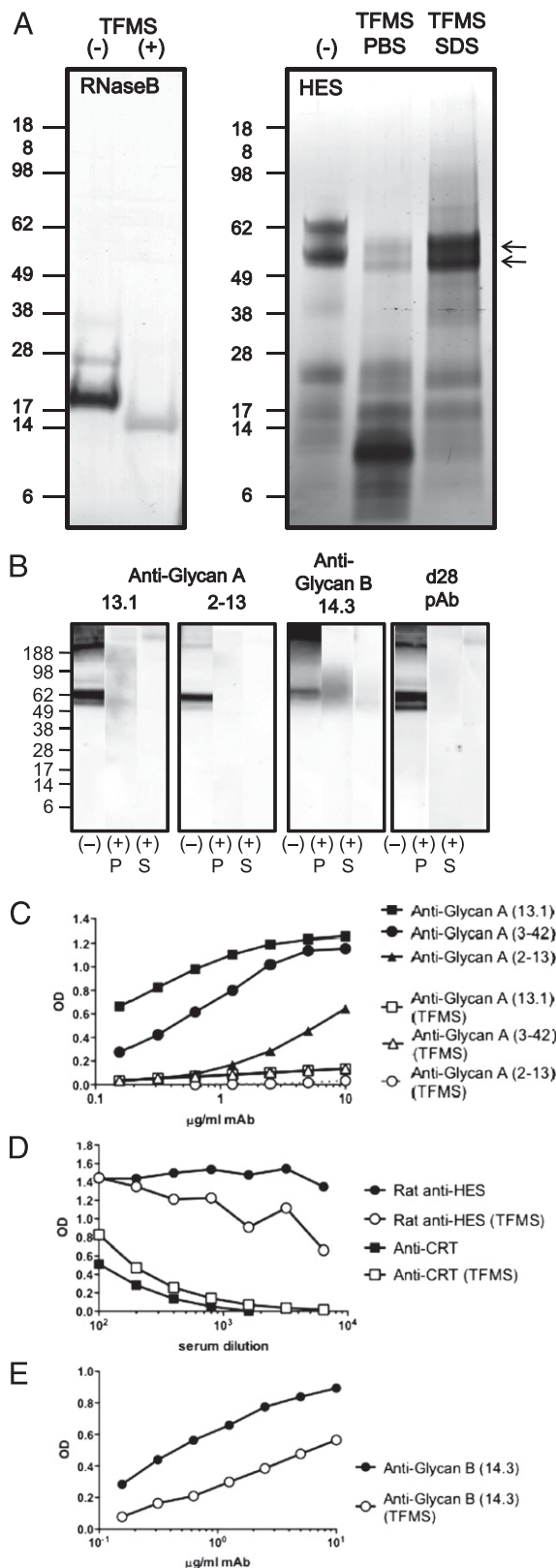


FIGURE 7. Chemical deglycosylation ablates anti-glycan A IgM Ab binding and reduces anti-glycan B binding to HM-65. *A*, Silver-stained one-dimensional SDS-PAGE of TFMS-treated (+) or mock-treated (-) HES and RNase B control glycoprotein. TFMS-treated HES was separated into PBS and SDS-soluble fractions. *B*, One-dimensional Western blots of mock-treated and TFMS-treated HES probed with anti-HES mAbs against glycans A and B and day 28 C57BL/6 polyclonal antiserum. *C*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES, used to measure binding of anti-glycan A mAb 13.1 (squares), 2-13 (tri-

angles), and 3-42 (circles). *D*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES probed with polyclonal rat anti-HES serum (circles) or rat Ab to recombinant *H. polygyrus* calreticulin (Hp-CRT; squares). *E*, ELISA of TFMS-treated (open symbols) and mock-treated (solid symbols) HES probed with the anti-glycan B (HM-65) mAb 14.3. The PBS-soluble fraction of TFMS was used for ELISA in *C–E*. Molecular mass markers are indicated in kilodaltons.

Glycan A is strongly represented on the adult cuticle, whereas glycan B is a somatic Ag

To localize glycan A and B epitopes within the adult parasite, we probed intact worms and sections by immunofluorescent microscopy. In sections, anti-glycan A mAb bound the worm surface, highlighting the longitudinal ridges of the cuticle (Fig. 9A). Anti-glycan A Ab also bound to the surface of intact adults in a pattern that similarly emphasized the ridges (Fig. 9B). At higher magnification, anti-glycan A Ab was seen to stain an ordered array of epitopes organized longitudinally along the cuticular furrows (Fig. 9C). In contrast, anti-glycan B Abs failed to bind to intact worms (Fig. 9D) while reacting strongly to somatic constituents in cross-sections (Fig. 9E); in particular, no cuticular binding was observed with anti-glycan B Ab.

To determine the macromolecules to which cuticular glycan A is conjugated, we surface radiolabeled adult worms and used mAbs to immunoprecipitate glycan A-bearing Ags. As shown in Fig. 9F, this procedure indicates that glycan A is expressed on at least four different molecular mass species, with a 180-kDa band predominating (Fig. 9F); only a small proportion of ~55-kDa VAL proteins are similarly immunoprecipitated. The conclusion that most surface glycan A is not borne on VAL proteins is supported by studies reported elsewhere that anti-VAL-1 and -VAL-2 mAbs bind only to localized areas of the cuticle (34). In contrast to glycan A, the glycan B-specific Abs were unable to immunoprecipitate radiolabeled surface components (Fig. 9F). A remarkable degree of cross-reactivity between ES and surface proteins was indicated as a polyclonal sera raised against HES was able to precipitate essentially all radiolabeled surface components (Fig. 9F).

Vaccination with HES confers protection against challenge whereas passive immunization with anti-HES mAb does not

Given the protective ability of VAL family members such as ASP in related helminth infections (45), we determined whether passive immunization with the anti-VAL protein and glycan mAbs were able to confer protection against challenge infection. We first wished to verify that adult worm-derived HES, in which VAL proteins are among the major Ags, could effectively vaccinate against challenge with larval parasites, as negative results have been reported in the literature (46). We found, however, that HES

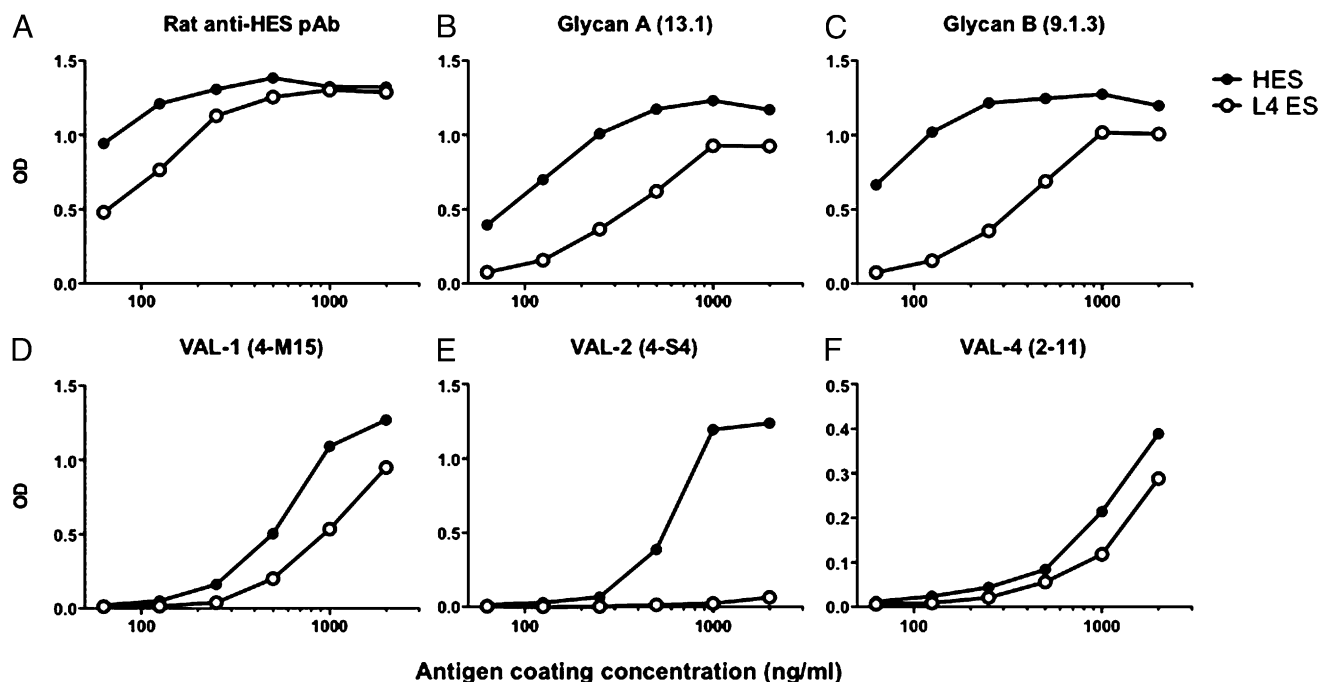


FIGURE 8. Monoclonal binding to ES from the tissue-phase L4 larvae show expression of glycans A and B, VAL-1 and VAL-4, but not VAL-2. A, ELISA reactivity of polyclonal rat anti-HES binding to plates coated with range of concentrations of adult HES (solid symbols) or L4 ES (open symbols) B, As above, with anti-glycan A mAb 13.1. C, As above, with anti-glycan B mAb 9.1.3. D, As above, with anti-VAL-1 mAb 4-M15. E, As above, with anti-VAL-2 mAb 4-S4. F, As above, with anti-VAL-4 mAb 2-11.

vaccination with alum adjuvant induced a potent humoral response, with titers of anti-HES IgG1 comparable to those seen following secondary infection (data not shown). Moreover, vaccinated animals had greatly reduced egg counts at day 14 than animals immunized with PBS-adjuvant alone (Fig. 10A), whereas by day 28, HES-immunized animals showed no fecal eggs and had expelled all adult worms (Fig. 10A, 10B). Thus, immune responses against adult secretions conferred highly effective and significant protection against larval challenge.

We next performed passive immunization experiments with each of the defined specificity mAbs given throughout the infection period. Using either 0.2 or 1 mg doses of mAb every 2–3 d, mice given anti-VAL-1, -VAL-2, or -VAL-4 IgG1 Abs showed no diminution in egg counts at day 14 (Fig. 10C) or day 28 (Fig. 10D) of infection, and indeed, anti-VAL-4 recipients showed elevated egg numbers in two independent experiments ($p < 0.05$ compared with recipients of MOPC31C). Furthermore, none of the anti-VAL mAbs induced worm expulsion as measured at day 28 (Fig. 10E).

In the same experiments, we also tested IgG1 anti-glycan B mAb for ability to passively protect recipient mice; however, as shown in Fig. 10C–E, this Ab also failed to reduce egg numbers or elicit expulsion of adult worms. Finally, IgM Ab against glycan A was tested, because no class-switched Abs of this specificity were observed. As with the other mAbs, anti-glycan A did not protect against egg production (data not shown) or worm persistence (Fig. 10F).

Discussion

The model system of *H. polygyrus* captures many essential characteristics of the gastrointestinal nematode infections that are highly prevalent in human and animal populations (47, 48). The parasite establishes a chronic infection, driving regulatory T and B cell subsets within a Th2-dominated environment (5, 25, 49–51) and altering innate populations such as dendritic cells (52, 53) and macrophages (54, 55). Immunity to *H. polygyrus* is slow to develop, particularly in the genetically most susceptible hosts (14),

but both B cells and the Abs they produce are important constituents of the protective immune response (4, 15, 16). In this study, we aimed primarily to define the Ags of adult parasites recognized by host serum Abs and secondarily to address whether those Ab specificities serve a protective function in the host–parasite relationship.

Previous antigenic analyses of *H. polygyrus* have involved a mixture of approaches and relatively simple characterization such as one-dimensional SDS-PAGE or column fractionation (32, 33, 56) or have investigated individual gene products that are postulated to play a role in immune recognition (26, 31, 57, 58). We have adopted in this study a more global approach to identify the major Ags recognized during primary infection, which we show are well represented in HES, although not in somatic extracts, presumably reflecting the fact that the immune system is exposed in a more continuous fashion to the secreted products of a luminal-dwelling live parasite. Indeed, we have detected glycan A in the serum of 7-d infected mice, demonstrating that products of a gastrointestinal parasite can disseminate to distant sites (J.P. Hewitson, unpublished observations). Ongoing work has also revealed that T cell Ags, driving the secretion of Th2 cytokines, are enriched in HES compared with somatic extract in a similar manner to serological Ags (J.P. Hewitson and K.J. Filbey, unpublished observations).

Murine Ab responses are known to be predominantly IgG1 with primary reactivity to secreted Ags in the 50- to 70-kDa range (14, 59). These Ags correspond to those we have now defined as VAL-1, VAL-2, and VAL-5 and appear likely to represent the products isolated from HES by Monroy et al. (57) to achieve a 40% reduction in egg production following vaccination. Interestingly, these glycoproteins bear a conserved antigenic carbohydrate we have termed glycan A, the structural analysis of which is now under way. Glycan A is also strongly associated with the cuticular surface of the adult worm and is partly responsible for the extensive degree of antigenic sharing between the secretions and

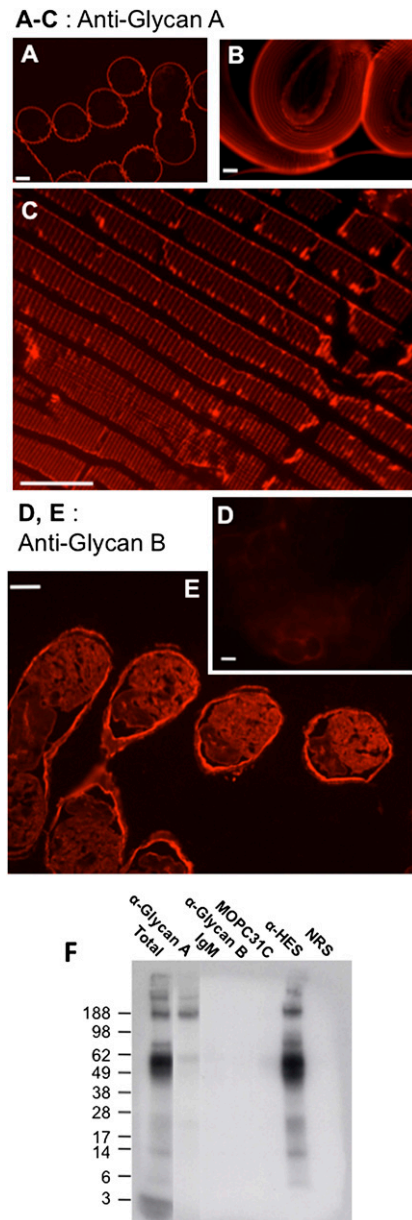


FIGURE 9. The immunodominant glycan A is present on the surface of adult worms. *A*, Binding of anti-glycan A mAb 13.1 to transverse sections of adult *H. polygyrus*. The corrugated cuticular ridges are visible. *B*, Binding of anti-glycan A mAb 13.1 to the surface of intact adult *H. polygyrus*. *C*, Binding of anti-glycan A mAb 13.1 to ordered structures within cuticular furrows on the surface of *H. polygyrus*. *D*, Failure of anti-glycan B mAb 14.3 to bind to intact worms. *E*, Binding of anti-glycan B mAb 14.3 to muscle layer and other structures in transverse sections of adult worms. *F*, SDS-PAGE of ^{125}I surface-labeled adult proteins immunoprecipitated with anti-glycan A mAb 13.1, revealed by autoradiography; similar analysis with anti-glycan B mAb 9.1.3 is also shown. Control IgM and IgG1 (MOPC31C) proteins are shown, as well as polyclonal rat anti-HES and normal rat serum (NRS). Scale bars (*A–E*), 100 μm . Molecular mass markers are indicated in kilodaltons.

surface of adult *H. polygyrus*, as reported by earlier investigators (33). This may indicate that HES, and the VAL components in particular, are shed from the surface of the worm or alternatively that molecules secreted in HES remain associated in some form with the parasite cuticle.

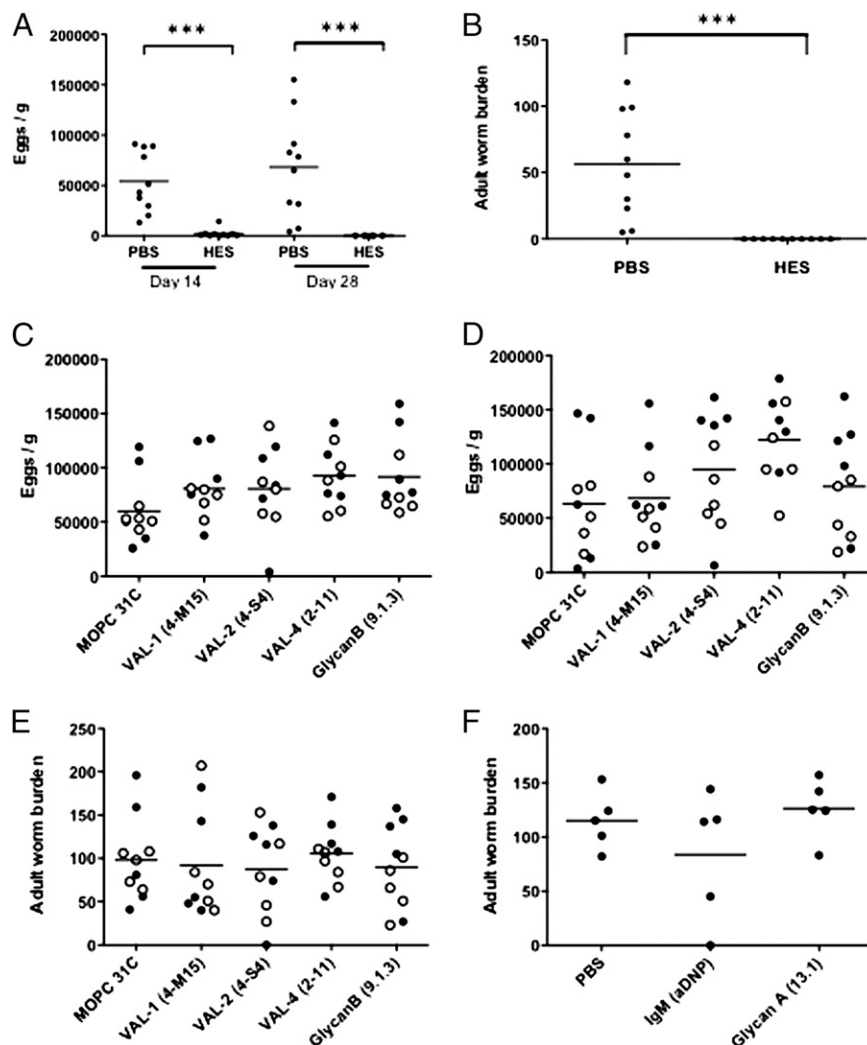
The VAL glycoproteins represent the immunodominant target of both IgM (against glycan A) and the class-switched IgG1 response

(against a conformational epitope, presumably the protein backbone). Most VAL proteins from other species have been reported to be glycosylated based on discrepancies between predicted and observed molecular mass [e.g., *Ancylostoma caninum* VALs (60)], although only *N*-glycans have so far been identified [on a VAL protein from the cattle nematode *Ostertagia ostertagi* (61)]. In this article, we show that a subset of *H. polygyrus* secreted VAL proteins are decorated with highly antigenic *O*-glycans, which are most likely concentrated in a serine/threonine-rich tract linking two conserved sperm coating protein (cd05380) domains. Similar stretches of predicted *O*-glycosylation can be observed in other nematode VAL proteins, including *Haemonchus contortus* Hc40 (accession number AAC03562; <http://www.ncbi.nlm.nih.gov/>), *Cooperia punctata* ASPs (AAK35199 and AAK35187), and *A. caninum* ASP-4 and ASP-6 (AAO63576 and AAO63578). It is also important to note that the VAL proteins themselves, rather than only their associated glycans, are likely to play a key role in host–parasite interactions, as *H. polygyrus* secretes a number of abundant nonglycosylated VAL proteins such as VAL-3 (34).

The nematode cuticle is an extracellular matrix assembled from specialized collagens and cuticlins (62), with sugar components that may vary greatly between species. For example, *Trichinella spiralis* conjugates a unique immunodominant glycan with a terminal tyvelose sugar onto multiple peptide backbones (63), providing a target for protective Abs (64, 65), whereas larvae of *Toxocara canis* (which invade the intestinal tract and migrate in tissues) release two unusually methylated and highly antigenic *O*-linked trisaccharides (66–68). Larvae of *Trichostrongylus colubriformis*, which, like *H. polygyrus*, is a member of the Trichostrongylid family, express a protease-resistant carbohydrate Ag that may act as the target of protective immunity (69). Another antigenic moiety closely related with helminth parasites is phosphorylcholine, which may be associated with high molecular mass proteoglycan-like molecules (30) or individual proteins that resolve on SDS-PAGE (41). Interestingly, both appear to be the case for HES in which PC is present on a high molecular mass product that does not stain for protein, as well as a 65-kDa component. Most intriguingly, mAbs to this combination (termed HM-65) do not react to PC itself, indicating that a distinct but as yet uncharacterized structure (provisionally named glycan B) is expressed. It remains possible that “glycan B” is not a true carbohydrate but a small haptenic group similar to diethylaminoethanol that is found in ES Ags of the filarial nematode *Litomosoides sigmodontis* (70). Hence, although we do not yet know the structural nature of glycans A and B, it is clear that *H. polygyrus* is not unusual in presenting extensive and immunodominant nonprotein specificities to the mammalian host.

Abs may exert a protective effect by several pathways (17); in particular, they may impede growth and migration during the histotrophic larval phase (20, 71), possibly by neutralizing key ES products (6), and may target the exposed epitopes on the surface of adult worms during the luminal phase. A major objective in studying Ab–Ag interactions in *H. polygyrus* is therefore to address whether particular specificities can confer immunological protection against infection. It is known that Abs play an important role in protective immunity to this parasite, as B cell-deficient animals suffer impaired immunity against secondary infection (4, 15, 16) as a result of absence of Ab as well as B cell participation in the cellular response. In tests of polyclonal serum Abs, the passive transfer of secondary infection sera can protect against challenge infection (15, 72), and this was associated with the IgG1 fraction (19). Although attempts to transfer immunity with primary infection sera have been less successful (15), purified IgG1 can reduce worm burdens and lead to stunting of adult parasites

FIGURE 10. Vaccination with adult HES confers sterile immunity to challenge, but passive immunization with mAbs from infected mice does not lead to worm expulsion. **A**, Fecal egg counts at 14 and 28 d postinfection in HES-alum vaccinated mice compared with PBS-alum alone. Each symbol represents a single mouse, and data shown are combined from two independent experiments. $***p < 0.0001$. **B**, Adult worm burdens at 28 d postinfection in HES-alum vaccinated mice compared with PBS-alum alone. Each symbol represents a single mouse, horizontal bars denote mean values, and data shown are combined from two independent experiments. $***p < 0.0001$. **C** and **D**, Fecal egg counts at 14 d (**C**) and 28 d (**D**) postinfection in mice receiving passive immunizations of anti-HES IgG1 mAbs or of MOPC31C control IgG1 Ab. Each symbol represents a single mouse, horizontal bars denote mean values, and data shown are combined from two independent experiments using 0.2 mg (open symbols) or 1 mg (closed symbol) mAb for each dose. **E**, Adult worm burdens at day 28 postinfection in the experiments shown in **C** and **D**. **F**, Adult worm burdens at day 28 postinfection in mice passively immunized with PBS alone, control anti-DNP IgM, or IgM anti-glycan A mAb 13.1. i.v. Each symbol represents a single mouse, and horizontal bars denote mean values; similar results were obtained in an independent experiment following i.p. delivery of mAb. Mice receiving i.p. injections of IgG1 Abs were assayed 24 h following the first transfer and were found to have specific anti-HES serum Abs equivalent to between 217 and 633 $\mu\text{g/ml}$ mAb (data not shown).



(20). This may imply that the efficacy of secondary serum Abs over primary sera reflects elevated titers of anti-worm Abs, rather than any difference in specificity induced by repeat exposure.

We therefore tested each of the mAb types generated in this study for their ability to confer protection by passive transfer. However, none exerted any effect on immunity. As we transferred considerable quantities of each, and these mAbs represent the major specificities present in primary infection sera, our data instead argue that the failure of primary sera to protect does not reflect a quantitative insufficiency in terms of concentration but either an inadequate affinity as a result of limited affinity maturation or, most interestingly, the absence of key new specificities that may arise only after multiple infections. The failure of mAb-mediated passive immunization contrasts with the sterile immunity generated following vaccination with HES that generates circulating anti-HES titers comparable to those seen following secondary infection. We also therefore examined the Ab profile following secondary infection, which is broadly similar to the primary but contains some additional specificities (such as the single-domain VAL-7 Ag). Future work will examine whether these are more effective targets of protective immunity. A further possibility to be tested is that combinations of Abs, for example, against each of the related VAL proteins, are required to neutralize a common function and that the full range of anti-VAL Abs are only generated through secondary infection.

An intriguing finding of this study is that the dominant anti-glycan A response, which is rapidly and extensively stimulated

by *H. polygyrus*, shows little protective capacity. This epitope may thus represent an example of a decoy Ag that is elaborated by the parasite to distract immune responses without risk of it inducing a lethal attack on the worm. It is also notable that despite strongly binding the adult worm surface, anti-glycan A Abs are not protective in vivo; this could reflect their restriction to IgM, suggesting that in the absence of class-switching Abs may not gain access to the intestinal sites of infection. The lack of other isotypes even after 28 d of infection suggests a deficiency in T cell help, potentially at the level of the TFH. As it is known that there are abundant IL-4-producing TFH in the draining MLN of *H. polygyrus* mice early in primary infection (73), it is surprising that no IgG response to glycan A is mounted, suggesting that this specificity is recognized and/or processed in an unusual manner.

The longevity of parasites can be attributed to their ability to evade or divert host immunity (74), and hence, the molecular basis of immune attack is of paramount interest. Our understanding of immune interactions with gastrointestinal helminths is primarily at the level of effector cell populations (39, 75) rather than identification of target molecules. At this time, the definition of parasite molecules in this important model of chronic gastrointestinal infection will provide an essential platform both to analyze the recognition of Ags and to identify the parasite products (proteins and sugars) that can modulate host immunity and facilitate protection (22). In this article, we have accordingly moved our definition forward from using crude parasite extracts to the more

antigenic and less complex HES, and finally to individual antigenic species, so that future work can study defined glycans and recombinant proteins. We have also established that most of the prominent Ags are secreted not only by the luminal-dwelling mature adult worms but also by the histotrophic larval stage, which is considered to be a major target of protective immunity. We can now begin to investigate how and where Abs act (21), the relative importance of functional neutralization and the recruitment of host effector cells, and the lethal mechanisms that achieve sterilizing immunity against intestinal helminths.

Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figures

Supplementary Figure 1

Alignment of amino acid sequences, after removal of signal peptides, of *H. polygyrus* VAL-1, VAL-2 and VAL-5 which are all recognized by IgM monoclonal antibodies subsequently show to bind Glycan A. Conserved residues are shaded; cysteine residues are highlighted in reverse shading. The nucleotide and amino acid sequences are deposited with NCBI under accession numbers JF914902 (VAL-1), JF914906 (VAL-2) and JF914911 (VAL-5).

Supplementary Figure 2

Predicted *N*- and *O*-glycosylation sites on VAL-1, -2, -3 and VAL-5. Signal peptides are shown in yellow highlighting; conserved domains with homology to sperm coating protein (SCP) are in gray highlighting, potential *N*-glycosylation sites are underlined in bold type; and predicted *O*-glycosylated Ser and Thr residues shown in red type. The nucleotide and amino acid sequences of VAL-3 are deposited with NCBI under accession number JF914909.

Supplementary Figure 3

ELISA reactivity of anti-HES mAb or day 28 C57BL/6 polyclonal serum to native HES (gray bars), PNGase A treated HES tryptic peptides (black bars) or mock treated HES tryptic peptides (white bars).

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10 20 30 40 50 60

VAL-1 (-SP) --- --TTC⁺PGNSGVTDAA⁺RTAALDAI⁺MEARRTA⁺VNGLNQNGATA⁺GTNL⁺PHGS⁺NMY⁺MLEWD⁺CN

VAL-2 (-SP) --- --QAAP⁺TCA⁺DA⁺ADKLEVER⁺--- --S⁺NVANAQPGY⁺GALVG⁺--- --S⁺Q⁺QMFAL⁺EC⁺DPL

VAL-5 (-SP) DGHRA⁺PPG⁺CT⁺DTA⁺IMD⁺DQER⁺EF⁺AYEQYWG⁺IR⁺TNL⁺VL⁺GN⁺YKD⁺--- --Q⁺GNVP⁺RKP⁺QNM⁺Y⁺VPT⁺YT⁺CS

70 80 90 100 110 120

VAL-1 (-SP) LEAL⁺AGV⁺HVPND⁺CSA⁺P-TAG⁺L⁺PN⁺NG⁺L⁺--- --SA⁺VVT⁺FA⁺PTT⁺ADA⁺VMT⁺QGVAS⁺WPVLLQTAANA⁺FA

VAL-2 (-SP) LEQAA⁺-IM⁺LVAL⁺CRD⁺PGL⁺PL⁺LACSSFNMYO⁺ALQG⁺QT⁺PLQADAL⁺TEAAIED⁺WRKKA⁺AFNP⁺---

VAL-5 (-SP) I⁺EN⁺AN⁺-LK⁺LADT⁺CPQT⁺-A⁺PT⁺FA⁺N⁺--- --YGLNT⁺QQAPL⁺DTA⁺SGYVADE⁺MTT⁺QIENV⁺T⁺-FA

130 140 150 160 170 180

VAL-1 (-SP) P⁺PAAG⁺T⁺PKY⁺E⁺GPTAGAALPADVLP⁺LC⁺HMIRG⁺ST⁺FKVC⁺C⁺VK⁺I⁺CTAQ⁺--- --AV⁺LV⁺CLY⁺D⁺-Q

VAL-2 (-SP) LPADA⁺IYSNQ⁺N⁺--- --LESF⁺ANM⁺I⁺YKS⁺TKVG⁺CA⁺YQS⁺HPA⁺LPPYPAAQSV⁺CV⁺FD⁺NK

VAL-5 (-SP) N⁺PYDGN⁺R⁺KY⁺E⁺--- --W⁺ANMAQATM⁺TRFG⁺CA⁺RA⁺SC⁺HTF⁺--- --EV⁺VI⁺CI⁺HD⁺-V

190 200 210 220 230 240 250

VAL-1 (-SP) PDL⁺K⁺QG⁺DTV⁺YDA⁺GTG⁺VQCNS⁺TADKL⁺CT⁺LYP⁺PT⁺CT⁺D⁺--- --I⁺YTGL⁺C⁺VKT⁺DV⁺T⁺--- --TT⁺TPSA⁺

VAL-2 (-SP) PALN⁺-SQLY⁺PGT⁺G⁺-TTG⁺CT⁺DPD⁺CAKAL⁺TGAT⁺CQTAAG⁺-NTQGL⁺CS⁺PQAGAA⁺F⁺TD⁺TT⁺PA⁺

VAL-5 (-SP) PD⁺IK⁺NG⁺QDI⁺Y⁺T⁺PGT⁺--- --P⁺VAN⁺TD⁺CT⁺TS⁺TPAV⁺C⁺D⁺DGL⁺CT⁺VI⁺YST⁺FPV⁺TSST⁺TP⁺L⁺-SS⁺TT⁺TA⁺

260 270 280 290 300 310

VAL-1 (-SP) TTTA⁺STLAV⁺FG⁺GA⁺GG⁺TGG⁺TGG⁺SR⁺--- --GTG⁺GT⁺GGT⁺GG⁺--- --AM⁺TR⁺CP⁺QNP⁺QNTD⁺DL⁺RY⁺LF⁺R

VAL-2 (-SP) TTTA⁺AA⁺T⁺AA⁺TT⁺TA⁺TT⁺TA⁺GP⁺TT⁺AD⁺--- --TT⁺TA⁺AA⁺TT⁺TA⁺APT⁺TTES⁺GL⁺MT⁺QT⁺IR⁺DR⁺VL

VAL-5 (-SP) A⁺TVTTS⁺TP⁺K⁺PGT⁺PA⁺PT⁺SS⁺SS⁺TS⁺AR⁺PST⁺TAPPAAV⁺TTN⁺ANP⁺GP⁺EL⁺NQW⁺CP⁺ANG⁺GML⁺DR⁺IR⁺SL⁺AL

320 330 340 350 360 370

VAL-1 (-SP) DMHNYR⁺KS⁺ET⁺AL⁺GR⁺TI⁺K⁺-NTGNY⁺LP⁺SS⁺SN⁺QY⁺NMY⁺SP⁺LEV⁺T⁺AI⁺HI⁺AST⁺CP⁺G⁺--- --SL⁺I⁺

VAL-2 (-SP) E⁺IN⁺NR⁺QL⁺AT⁺GO⁺VN⁺GR⁺TG⁺Q⁺N⁺CG⁺TG⁺M⁺I⁺YQ⁺MKT⁺DRG⁺LEV⁺LAQNYAD⁺QC⁺PTNANGS⁺AVATRP

VAL-5 (-SP) DMHNYR⁺RS⁺QLA⁺KGL⁺VA⁺K⁺-NNGNL⁺LP⁺FAA⁺ANM⁺K⁺NBY⁺DC⁺LEKA⁺AQA⁺HAD⁺GV⁺GGDS⁺DP⁺AT⁺RP⁺GI

380 390 400 410 420 430 440

VAL-1 (-SP) QSG⁺RLIGQV⁺PI⁺GA⁺YT⁺TT⁺AT⁺QDI⁺VKS⁺L⁺M⁺RV⁺YR⁺QVNG⁺P⁺GMQYTF⁺RA⁺QH⁺VGT⁺PIA⁺--- --S⁺FTQMAWA

VAL-2 (-SP) DNGENV⁺KI⁺IPSN⁺TV⁺PFY⁺DAVLSASQSW⁺DEI⁺-AT⁺EGV⁺NS⁺QMR⁺FTDFLQTK⁺PLA⁺PIR⁺WTQMAWA

VAL-5 (-SP) QENVHS⁺VY⁺IS⁺QS⁺T⁺PYRM⁺DAMRES⁺VK⁺QWMS⁺QV⁺RK⁺VEG⁺IGMA⁺VTF⁺RES⁺HLQST⁺IQ⁺--- --W⁺FTKMAWA

450 460 470 480 490 500

VAL-1 (-SP) ASR⁺RLG⁺CA⁺VA⁺RC⁺PT⁺--- --AY⁺IA⁺V⁺CNYE⁺PI⁺GNIV⁺G⁺Q⁺IY⁺TP⁺GT⁺FC⁺TA⁺--- --CT⁺YGS⁺T⁺CT⁺ATQGL⁺CT

VAL-2 (-SP) TTY⁺RLG⁺CA⁺GV⁺QC⁺GG⁺--- --NTV⁺V⁺CRYA⁺PRGNIV⁺D⁺Q⁺IY⁺KV⁺GAP⁺CG⁺DC⁺MSS⁺CI⁺SGSL⁺CT⁺PP⁺LRSL⁺CT

VAL-5 (-SP) TTE⁺NLG⁺CA⁺VT⁺K⁺NSNG⁺N⁺FI⁺V⁺CRYK⁺PG⁺GNIV⁺N⁺EV⁺V⁺YK⁺GAC⁺Q⁺K⁺--- --CP⁺AGYS⁺CS⁺TDEK⁺LC⁺CI

VAL-1 (-SP) LP-

VAL-2 (-SP) ---

VAL-5 (-SP) VIS

VAL-1 (18 x potential O site/ 1 x N site)

MWLPLILLSAVANREGTATTCPGNSGVTDAA**RTAALDAINEARRTAVNGIMQNGATA**
 GTNLPHGNSMYMLEWDCNLEALAGVMVPNDCSAPTAGLPNNGLSAAVV**TPAPTTADA**
 VMTQGVASWPVLLQTAANAFAPPAAG**TPVKYEGPTAGAALPADVPLGNMIRGSTTK**
 VGCTVKICTAQAVLVCLYDQPDLKQGDVYDAGTGVCQ**NSTADKLC**TLYPPPTCDIY
 TGLCVK**TDVTTTTTPSA****TTTAST**LAVFPGGAGG**TGGTGGSRGTGGTGGTGGANTRCP**
 QNPQMTDDLRYLFRDMHNYRRSETALGRTIKNTGNYLPSSSNMQYMRYSCTPLEVTAI
 HIASTCPGSLIQSGRLIGQVPIGAYTFTTATQDIVKSLWRVVRQVNGPGMQVTFKAQ
 HVGTPIASFTQMAWAASRRLGCAVARCPTAYIAVCN**YEP**IGNIVGQQIYTPGTPCTA
 CTYGSTCTATQGLC**TL**P

VAL-2 (26 x potential O site / 1 x N site)

MKRIIILFLVAGLIHRGNAQAAPTCADVA**DKLNEVRSNVARAQPGYGALVGSQQMFA**
 LECDPLLEQAAIMLVALCRDPGLPLLAGSSFNMYQALQGGTPLQADALIEAAIEDWR
 KKAAFNPLPADAIYSNQLESFANMIYYKSTKVGCAYQSRFALPPYPAAQSVVVCVFD
 NKPALNSQLYPPGTGTGCTDPDCANALTGATCQTAAGNTQGLCSPQAGAAFP**TDTT**
 PAA**TTTTAAATTTAA****TTTAA**TTAGP**TTAD****TTTTAAATTTA**AP**TTTES**GLMTQTI**RDRVL**
 EIHNNRRQLLATGQVVGRTGQNCGTGMNIYQMKYDRGLEVLQNYADQCP**TNANGS**
 AVATRPDNGENVKIIPSNTVPFYDAVLSASQSWDEIAINGVNHQMRFTDFLQTKPL
 APIRWTQMAWATTYRLGCGVQRCGGNTVVVCRYAPRGNIVDQYIYKVGAPCGDCMSS
 CISGSLCTPPLRSL**S**

VAL-3 (2 x potential O site / 1 x N site)

MRPIMWAILIVLGASTPSASA**ADYGCKQSLRTDDES****RDFVLLFHNVFRQNIALNLQSY**
 NFGKGLGPASNMYELEWDCKLEEAEEKIADTCDEKATDVYAQNGVKIRGDTRYSAM
 SNFIDAMTMTGDLWFYTGITITNVFKDANLS**IIANMAYS**SKTTKVGC**SYNWC**DTNKFF
 AVCLYNRLGNQLDNVLWEEGKHCKMDGECTTYAGSTCNSQGLCVDAAHADDVYEGEE
 KEEIQPPAEVIVPNPEGIIGDCP**TDNGMTNEI****RMSLLDKHNGYRTL**VAKGEAENPLG
 SSTGYAPKAARMRMVYDCNIEVNAMKNAAGCTYGHSSNNADRPGLGENI**WALWGPAN**
 YPLVKAEEAATDMWFSELKDPGMPEDN**ILTLAVVNR**PKAIGHYTQIVWQSTYHLGCG
 LKNCPDNMCYAVCQYGPAGNYLNSLVYEIGDPCNVDSCKCIGCKCSKEEAMC**ILPP**
 SLPWTA**TDGILPA**

VAL-5 (30 x potential O site / 1 x N)

MLSQITVLLHAAVYRLTSA**DGHRAPFGCTDTAIMDDQE****REFAYEQYWGIRTNLVLG**
 NYKDQQGNVPPKQNMVPTYTCSIEEMALKLADTCPQ**TAP**TPANYGLNYQQAPLD
 TAIGYVADEWTTQIE**NTFAN**PYDGNPKYKEWANMAQATMTRFGCARASCTTFEVVI
 CIHDVDPDIKNGQDIYTPGTPCVAHTDCTTSTPAVCDDGLCYVIY**TTPVSTSS****TTPLS**
STTSAA**TVTTST**TPKGP**TPAP****TSSSTS**ARP**STT**APPA**AVTT**NANPGPELNQWCPAN
 GGMLDRI**RS**LALDMHNYRRSQLAKGLVAKNNGNLLPPAANMRKMRYDCELEKAAQAH
 ADGCVGGSDPATRPGIQENVHVS**YISQSTPYRMDAMRESVKQWSQVRK**VEGIGMA
 VTFKEHHLQSTIQWFTKMAWATTEWLGCAVTKCNSNGNF**IIVCRYK**PGGNIIVNHVVY
 EKGAQCKCPAGYSCSTDEKLCIVIS

