Human Immunoglobulin G Mediates Protective Immunity and Identifies Protective Antigens against Larval Strongyloides stercoralis in Mice

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Protective immunity to larval Strongyloides stercoralis in mice has been shown to be dependent on antibody, complement, and granulocytes. The goals of the present study was to determine the following: (1) whether human serum could passively transfer immunity to mice, (2) the mechanism by which the serum mediated killing, and (3) whether the antigens (Ags) recognized by the protective human antibody could induce protective immunity in mice. Immunoglobulin G (IgG) from a S. stercoralis–seropositive individual passively transferred immunity to mice. The antibody required granulocytes, but not eosinophils, and complement activation to kill the larvae. Antibody-dependent cellular cytotoxicity was not required for larval killing. Immunization of mice with soluble larval Ags isolated by use of the protective immune IgG resulted in protective immunity. In conclusion, immunity could be transferred to mice by IgG from immune humans, and Ags identified by the immune human IgG induced protective immunity in mice, which thereby suggests their possible use in a vaccine against this infection.

Strongyloides stercoralis, an intestinal parasitic nematode of humans, is found worldwide and produces chronic but usually asymptomatic infections. Steroid or human T lymphotrophic virus type 1 (HTLV-1)–induced immunosuppression of S. stercoralis–infected individuals can lead to hyperinfection, which suggests that the disease is under immunological control [1]. Antibodies may have a central role in protective immunity against this infection on the basis of the observation that hypogammaglobulinemia has been associated with refractory cases of strongyloidiasis [2]. Evaluations of human antibody responses against soluble S. stercoralis larval proteins have observed that IgE [3, 4], IgA [4, 5], and IgG [6] were detected in S. stercoralis–infected individuals and that each of these antibody isotypes has been proposed as a possible mediator of immune protection. IgG from S. stercoralis–infected individuals also has been useful in identifying larval antigens (Ags) for use in immunodiagnosis [7–9]. In particular, recombinant Ag 5a (r5a) was identified from an infective third-stage larvae (L-3) cDNA library by human IgG and was recognized by all tested patients who had parasitologically proven S. stercoralis infections. r5a was not recognized by patients infected with a variety of nematode and trematode infections [10].

The immune response in humans to S. stercoralis appears to be Th2 mediated. Patients coinfected with S. stercoralis and HTLV-1, a Th1-inducing infection, have decreased parasite-specific IgE and interleukin (IL)–5 responses to S. stercoralis [11, 12]. This observation implies that the Th1 response induced by HTLV-1 reduces the Th2 response to S. stercoralis. The suppression of the antihelminth Th2 immune response
results in a reduction in hypersensitivity reactions observed in skin tests with larval proteins [13]. Furthermore, coinfection with HTLV-1 has been associated with the development of S. stercoralis hyperinfection [14–16], which proved to be fatal in some patients [17].

Protective immunity in BALB/cByJ mice against S. stercoralis L-3, elicited with live larvae, was shown to be dependent on either IgM or IgG in association with complement and granulocytes [18, 19]. In addition, IgG mediated protective immunity by means of an antibody-dependent cellular cytotoxicity (ADCC) mechanism [20]. The development of protective immunity in mice required IL-4 and IL-5, thereby showing a dependency on a Th2 response. Mice lacking IL-5 developed a decreased antibody response that did not result in larval killing [21–23]. Altering the immune response in mice from a Th2 to a Th1 response by administering recombinant IL-12 resulted in the loss of protective immunity [22]. Therefore, the data from human and mouse studies indicate that the establishment of a Th2-type immune response is required for control of S. stercoralis.

Ags capable of inducing protective immunity against larval S. stercoralis have been identified in mice. Analysis of various soluble larval protein pools demonstrated that mice immunized with sodium deoxycholate-soluble (DOC) L-3 proteins developed a protective IgG-mediated immune response. IgG from mice immunized with the protective DOC Ags was used to purify a small number of larval proteins, which were called mouse IgG-specific Ags (mouse-IgG-Ags), by use of affinity chromatography. Immunization of mice with mouse-IgG-Ags resulted in higher levels of protective immunity than that seen in mice immunized with the larger DOC Ag pool from which they were derived. In addition, mice immunized with DOC Ags or mouse-IgG-Ags have increased eosinophil levels and IL-5 production [24], which supports the concept that immunization of mice with either live worms and soluble larval Ags induces a similar protective Th2 response.

The aim of the present study was to demonstrate that serum from humans exposed to S. stercoralis could passively transfer immunity to naive mice. The specific human antibody isotype active in protective immunity was identified, and the mechanism used by the protective antibody to kill the larvae was determined. Finally, Ags from the DOC Ag pool specifically recognized by the protective human antibody were purified; this limited pool of Ags identified was shown to induce high levels of protective immunity in mice.

**MATERIALS AND METHODS**

**Animals and parasites.** Wild-type (wt) BALB/cByJ and C57BL/6J mice were obtained from Jackson Laboratory. FcγRII γRII −/− × FcγRIIB −/− double-knockout (KO) mice, which lack all Fc receptors for IgG [25, 26], and control wt C57BL/6 × 129/Sv F2 mice were purchased from Taconic. IL-5−/− mice, which were a generous gift from Manfred Kopf (Basel Institute for Immunology, Basel, Switzerland) [27], and C3−/− mice, which were obtained from Jackson Laboratory, both on a C57BL/6 background, were bred at Thomas Jefferson University (Philadelphia). All mice were housed in filter-top microisolator boxes under light- and temperature-controlled conditions. Male mice, aged 6–8 weeks, were used for all experiments. S. stercoralis L-3 were obtained from the cultures of fresh stools from a laboratory dog infected with the parasite, as described elsewhere [18]. Larvae were collected from 5–7 day charcoal cultures and were washed and resuspended in culture medium.

Diffusion chambers covered with 2.0- or 0.1-μm Isopore membranes (Millipore) were constructed, as described elsewhere [18]. Challenge infections consisted of 50 L-3 placed in each diffusion chamber that were subcutaneously implanted on the dorsal surface of the mouse for 24 or 96 h. Parasites were considered to be vital on the basis of motility and morphology. Cells found within diffusion chambers were centrifuged onto slides and then were stained for differential counts.

**Serum transfer of protective immunity.** In the present study, 2 sources of human serum samples were used for transfer of antibody into mice. First, a pool of human serum samples was obtained from 5 patients confirmed to be infected with S. stercoralis by identification of larvae in stool samples, which is referred to as “infected serum.” Pooled serum samples from uninfected individuals was used as naive serum. Second, outdated plasma samples that were obtained from a Haitian blood bank were screened for reactivity with recombinant S. stercoralis L-3 r5a [10], and 1 sample was identified as seropositive. The seropositive plasma sample was obtained from an anonymous donor; therefore, S. stercoralis infection status was unknown, although the blood tested negative for circulating filarial Ags. The plasma was treated with 5 U of thrombin (JPI Jones Daniels Pharmaceuticals) at 37°C for 30 min to obtain serum. This serum sample is referred to as “immune serum.” A serum sample obtained from a seronegative individual was used as naive serum.

Serum samples from humans and from mice immunized with DOC Ags were passed through a Fast Flow Protein G column (Sigma) to obtain a purified IgG fraction. The IgG fraction was eluted by use of a 0.5-mol acetic acid (pH 3.0), and the eluent was immediately neutralized with concentrated Tris-base (pH 9.8). IgG concentrations before and after fractionation were determined by use of ELISA.

Whole serum or the purified IgG fraction was injected into mice at the time of challenge into the subcutaneous pocket surrounding the implanted diffusion chamber. The amount of IgG transferred was the same quantity found in 100 μL of either unfractionated control or immune serum, as determined by ELISA, and was diluted at a ratio of 1:1 in sterile PBS. For all serum transfers, challenge infections consisted of 50 live L-3
contained within diffusion chambers with recovery 24 h after challenge. Mice were treated with monoclonal antibody (MAb) RB6-8C5 to eliminate granulocytes [28] by use of the following protocol: 0.5 mg was injected intraperitoneally 3 days before challenge infection and on the day of challenge.

**Soluble L-3 Ag preparation and affinity chromatography.** DOC-soluble *S. stercoralis* Ags were prepared, as described elsewhere [24]. Immune and naive IgG affinity columns were prepared by linking purified IgG to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech AB). To obtain human IgG-specific Ags (human-IgG–Ags), DOC Ags were first passed through the naive IgG column to remove larval proteins not specifically binding to IgG [29] and then were passed through the immune IgG column. Proteins were eluted with 0.5-mol acetic acid (pH 3.0), immediately neutralized with saturated Tris-HCl, and dialyzed against PBS.

**Western blot analysis and silver staining.** Larval Ags and protein standards were separated by one-dimensional SDS-PAGE by use of 12% polyacrylamide slab gels. After electrophoresis, gels were prepared for either Western blot or silver staining. For Western blot analysis, proteins were transferred to nitrocellulose (BioRad). The membrane was blocked with a solution of 5% (wt/vol) Carnation brand nonfat dry milk (Nestle Food). Membranes were incubated with purified naive and immune IgG or IgG from DOC Ag–immunized mice. Membranes were incubated with anti–human IgG HRP-conjugate antibody (PharMingen) or anti–mouse HRP-conjugate antibody (PharMingen). Western blots were developed by use of the Enhanced Chemiluminescence (ECL; NEN Life Sciences Products) reagent and were visualized by use of Biomax ML Kodak film (Eastman Kodak). For silver staining, gels were developed by use of the Silver Stain Plus Kit (BioRad).

**ELISA.** To measure human IgG in the serum samples and column eluents, Nunc Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with 2 μg/mL goat anti–human IgG (PharMingen). Plates were blocked with borate-blocking buffer solution (BBS), and test samples were diluted in BBS and placed in duplicate wells at serial dilutions. Appropriately matched biotinylated goat anti–human IgG Abs were added to the wells, which was followed by the addition of avidin peroxidase (Sigma) and then peroxidase substrate 2,2’-azinodi (3-ethylbenzthiazoline-6-sulfonate) (ABTS; Kirkggaard & Perry Laboratories).

**Immunization of mice with soluble L-3 Ag.** Mice were immunized with Ags obtained from the DOC Ag pool in a 1:20 dilution of 2% aluminum hydroxide (alum) low viscosity rehydragel (Reheis). Mice were immunized with 25 μg of DOC Ags, 20 μg of nonbound DOC Ags, and 10 μg of human-IgG-Ags in 200 μL of the Ag/alum mixture on day 0 and day 14. On day 28, mice were challenged with L-3 that were contained within diffusion chambers, which were recovered 96 h after challenge.

**Electron microscopy.** L-3 were fixed for 30 min in 0.25% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) containing 1% sucrose and then were processed for immunoelectron microscopy. Thin sections of embedded larvae were incubated with purified IgG from a seropositive individual and a naive individual, which was then followed by incubation with a suspension of 15-nm gold particles coated with protein A, as described elsewhere [30].

**Statistical analysis.** Experiments consisted of 5 mice/group, unless otherwise noted. Experiments were performed at least twice, and consistent results were obtained from the repeated experiments; therefore, data are shown from 1 representative experiment. Statistical analysis of the data was performed in Systat (version 5.2) by use of the MGLH multifactorial analysis of variance.

**RESULTS**

**Passive transfer of immunity with human IgG into mice.** Experiments were performed to determine whether human serum pooled from 5 *S. stercoralis*-infected individuals could transfer immunity to mice. The human serum was transferred at the time of the challenge infection by injecting the serum into the subcutaneous pocket surrounding the implanted diffusion chamber. Recovery of larvae from the diffusion chambers 24 h after implantation revealed that the transfer of pooled serum from *S. stercoralis*-infected humans resulted in a 73% reduction in larval survival (figure 1A). Transfer of the IgG fraction from the pooled serum resulted in a 35% decrease in larval survival, whereas the combined IgA, IgE, and IgM fraction provided no protection, which demonstrates that IgG from *S. stercoralis*-infected individuals is the protective antibody isotype in mice.

A serum sample obtained from an *S. stercoralis*–seropositive individual also was used in passive transfer experiments. Before injection, the serum was fractionated into an IgG fraction and a combined IgA, IgE, and IgM fraction. Passive transfer of the unfractionated immune serum and the immune IgG fraction into naive mice resulted in a 95% reduction in parasite survival (figure 1B). The combined IgA, IgE, and IgM fraction did not transfer protective immunity to mice. Cells found within the implanted diffusion chambers, after transfer of either the naive or immune antibody fractions, were 92% ± 7% neutrophils, 7% ± 7% macrophages, and 0.4% ± 0.6% eosinophils. Therefore, IgG from *S. stercoralis*–infected individuals or the *S. stercoralis*–seropositive individual could transfer immunity against *S. stercoralis* to mice. However, there was no association between immunity and the elevation in any cell type found in the microenvironment of the parasite. Because equivalent results were obtained with the human serum pool and the single seropositive serum sample in
Figure 1. Passive transfer of human IgG from the pooled 
Strongyloides stercoralis–infected serum samples (A) and the seropositive individual (B) confers protective immunity to naive mice. Data are mean ± SD of 5 animals/group. *P < .05, vs. naive serum.

mice, all subsequent experiments were performed with the more readily available seropositive serum sample.

Role of complement and cells in larval killing by human immune IgG. Passive transfer of immune IgG into naive C3−/− mice was performed to determine whether complement activation was required for larval killing. C3−/− mice lack the C3 component of the complement cascade, which blocks activation of the classical and alternative complement pathways. Immune IgG did not transfer protective immunity to C3−/− mice, which indicates that immune IgG mediates larval killing via complement-dependent mechanisms (figure 2A). To determine whether cellular contact with L-3 was required for larval killing, diffusion chambers were constructed by use of 0.1-μm pore-size membranes that block cellular infiltration while still allowing for free passage of soluble immune factors, including complement. Larvae implanted within 0.1-μm pore-size membrane diffusion chambers were not killed by the immune IgG at the level seen for larvae implanted within 2.0-μm pore-size membrane chambers (figure 2B). These results indicate that larval killing by immune IgG primarily operates by use of a mechanism dependent on cellular contact with the larvae.

Mice were treated with MAB RB6-8C5, which depletes eosinophils and neutrophils, to determine whether granulocytes were required during immune IgG-mediated killing of L-3 in mice. Elimination of granulocytes blocked the ability of the immune IgG to transfer immunity (figure 3). Differential cellular analysis of the diffusion chamber contents indicated that neutrophils and eosinophils were eliminated from the animals treated with MAb RB6-8C5 and left only a small number of macrophages within the diffusion chambers (data not shown).

To distinguish between the requirement for neutrophils and eosinophils in larval killing, immune IgG was transferred into IL-5−/− mice, which are deficient in eosinophils, but still have wt levels of neutrophils. Immune IgG killed L-3 equally well in wt and IL-5−/− mice (figure 4A), which suggests that the effector cells are neutrophils. To determine whether the immune IgG and the mouse neutrophils killed the L-3 via an ADCC mechanism, immune IgG was passively transferred into FeRγ−/− × FeγRIIB−/− mice. Passive transfer of immune IgG into wt and FeRγ−/− × FeγRIIB−/− mice resulted in equivalent levels of protection (figure 4B), which indicates that mouse cells were not binding to immune IgG to mediate killing. Thus, the larval killing mechanism of immune IgG in mice was not mediated by the ADCC mechanism.

L-3 Ags recognized by human IgG. Fixed L-3 were sectioned and then incubated with the protective immune IgG to determine the ultrastructural localization of the immune IgG targets. Immune IgG bound to the surface and the internal

Figure 2. Immune IgG-mediated killing requires complement and cellular contact. Immune IgG was passively transferred to C3−/− mice (A) and to mice implanted with diffusion chambers constructed with 0.1-μm pore-size membranes to prevent cells from entering the diffusion chamber (B). Data are mean ± SD of 5 animals/group. *P < .05, vs. control treatment.
components of the cuticle and to the glands surrounding the esophagus. Weak binding of the immune IgG was seen in the muscle, coelomic cavity, and nerve cords (figure 5).

Western blot analysis was performed to determine which proteins from the DOC Ag pool were recognized by immune IgG. The analyses revealed that, at the same dilution, naive human IgG did not bind to any larval proteins, whereas immune IgG recognized ~18 proteins in the range of 20–150 kDa (figure 6, lane 1). The number of recognized proteins probably is an underestimation, because a one-dimensional Western blot analysis was performed. Analyses also were performed to compare the Ags recognized by the human immune IgG and Ags recognized by protective IgG from mice immunized with DOC Ags. Approximately 4 proteins were recognized by both protective IgG antibodies, which suggests that the mouse and human IgG antibodies target different larval proteins within the DOC Ag pool (figure 6, lane 2).

DOC Ags were passed over affinity columns constructed with naive and immune human IgG to purify the Ags specifically recognized by the immune IgG. The proteins were first passed over a column made with naive human IgG, to remove any proteins that might not specifically bind to human IgG. The antigens that did not bind to the naive IgG column then were passed through an affinity column constructed with human immune IgG. The proteins eluted from this column, termed human IgG-specific Ags (human-IgG-Ags), were run on a 12% polyacrylamide gel and were silver stained. The human immune IgG (figure 7, lane 4) was run as a control to distinguish between the eluted Ags and any IgG disassociating from the column. One-dimensional silver stain analysis revealed that ~13 proteins were eluted from the immune IgG column (figure 7, lane 5). Specifically, the molecular weights of the isolated proteins were 115, 98, 85, 76, 67, 65, 58, 45, 35, 34.5, 32, 31, and 25.5 kDa. The 65–67-kDa and 45-kDa bands, which were faint in the DOC Ag lane (lane 2), became highly visible in the affinity-column isolated Ags (lane 5). In addition, many bands located in the 20–36-kDa range in the DOC Ag lane were not found in the affinity-column isolated Ags (lane 5), but were found in the nonbound Ag lane (lane 3).

Mice were immunized with the DOC Ags, human-IgG-Ags, and proteins that did not bind to the immune IgG column (nonbound DOC Ags). All these proteins were administered with alum as the adjuvant. Mice immunized with human-IgG-Ags had a 76% reduction in larval survival, mice immunized with the entire pool of DOC Ags had a 52% reduction of larval survival, and mice immunized with the nonbound DOC Ags had a 54% reduction of larval survival (figure 8A). Differential cellular analysis of diffusion chamber contents revealed that eosinophil counts increased after immunization with all 3 larval protein pools (figure 8B), whereas neutrophil counts were equally represented in all groups (data not shown).

**DISCUSSION**

The aim of the present study was to gain an understanding of human protective immunity against larval *S. stercoralis*. Transfer into mice of purified IgG derived from a pool of serum from *S. stercoralis*-infected individuals and purified IgG from a seropositive individual both resulted in significant reductions in...
larval survival. Xenogeneic transfer of antibodies into mice to demonstrate protective immune responses against helminths has been described elsewhere [31–34]. The individual sero-positive serum sample was used for further analysis of the immune response in the present study, because it was shown to confer a comparable protective response, as seen with the pooled serum from several *S. stercoralis*-infected individuals.

IgG was the only antibody isotype from the pooled or the seropositive human serum that could transfer protective immunity to mice. The specific subclass of IgG that was protective was not determined, although ELISA data indicated that only 1% of total IgG response to the soluble L-3 Ags was by IgG4; the remainder was made up of equal responses by IgG1, IgG2, and IgG3 (authors' unpublished data). Analysis of the antibody-isotype responses of *S. stercoralis*-infected individuals has revealed a variety of elevated antibody isotypes. These include high levels of parasite-specific IgG [6], IgE [3], and IgG4 [35], and elevated levels of IgA associated with an absence of larvae in the feces of chronically infected individuals [5]. Three different types of mouse antibodies have been shown to be protective against *S. stercoralis* in mice: IgG from mice immunized with DOC Ags and IgM and IgG from mice immunized with live L-3 [19, 20, 24]. Furthermore, protective immunity in rats to the nematode *Strongyloides ratti* also was dependent on IgG [36]. Therefore, it is clear that IgG from a variety of sources is protective against larval *S. stercoralis*.

L-3 implanted in mice in diffusion chambers that blocked cell entry were not killed by the immune IgG, which demonstrates that cell contact was required for the IgG to kill L-3. Treatment of mice with a MAb to eliminate granulocytes resulted in the ablation of protective immunity that is dependent on immune IgG. Furthermore, transfer of human IgG to IL-5−/− mice resulted in protective immunity, which indicates that eosinophils were not required as effector cells and suggests that neutrophils were the active cells in the killing process. Protective IgG [20] and IgM [21] recovered from mice immunized with live L-3 also transferred immunity into IL-5−/− mice, which confirms that neutrophils were the probable effector cells in mice functioning with antibody from 3 different sources. However, previous results have shown that a Th2 response and eosinophilia are required for the induction of a protective immune response against *S. stercoralis* in mice [21, 22]. Th2 responses and eosinophilia are also common findings in strongyloidiasis in humans [37–40] and are associated with an improved disease prognosis [12, 41].
Figure 6. Western blot analysis comparing the antigens (Ags) recognized by mouse IgG from mice immunized with sodium deoxycholate-soluble (DOC)–soluble larval Ags and human immune IgG. Lane 1, mouse IgG–recognized Ags; lane 2, human IgG–recognized Ags. Naive IgG, diluted to the same concentration as immune IgG, from the mouse and human did not visibly recognize any larval Ags.

Therefore, eosinophils may be required for the induction of the protective immune response in humans, as they are in mice. The present study suggests that neutrophils, not eosinophils, are essential participants in antibody-dependent killing of *S. stercoralis* L-3 in mice.

Human IgG was shown by immunoelectron microscopy to bind to the surface of the larvae, which suggests that the mechanism by which the antibody and cells killed the larvae would be ADCC. Mouse Fc receptors can bind to human IgG with a relatively high affinity [42], which makes this mechanism possible. However, experiments demonstrated that human IgG transferred to mice lacking all Fc receptors could still kill the challenge larvae. This observation differs from the results obtained by use of IgG purified from mice immunized with live L-3, from which it was shown that the larvicidal activity of the antibody was lost if transferred into mice lacking Fc receptors for IgG [20]. Human IgG did not transfer protective immunity to C3−/− mice, which indicates that C3 is required for larval killing. Thus, although both mouse and human IgG require complement and granulocytes to mediate larval killing, they differ in the requirement for ADCC-mediated larval killing in mice. A possible mechanism whereby human IgG mediates larval killing in mice is by binding to the surface of the larvae, which thereby activates the complement cascade. Granulocytes are then recruited by C3a, bind to C3b, and degranulate, which causes damage to the larval surface and result in the death of the worm. Evidence that cells can attach to the larval surfaces via complement comes from studies showing that human polymorphonuclear cells and monocytes bound to *S. stercoralis* L-3 [43] and that mouse eosinophils attach and kill *Nippostrongylus brasiliensis* larvae through complement on the surface of the worm [44].

Mice immunized with DOC L-3 proteins develop protective immunity that is dependent on IgG [24]. The IgG binds to the muscles and nerve cord [24], and the mechanism of action of this mouse IgG was shown to be ADCC independent [20], which is similar to that of the human IgG used in the present study. In addition, IgG from mice immunized with live L-3 recognizes the coelomic cavities and the hypodermis [20], which are clearly different than the larval structures that the human IgG targets. Western blot analyses comparing the Ags recognized by the 2 protective mouse IgG and the human IgG revealed few commonly recognized Ags and many uniquely recognized Ags [20], which thereby supports the immunoelectron microscopy observations. Mice immunized with the purified mouse-IgG-Ags developed protective immunity at higher levels, compared with that seen in mice immunized with the larger DOC Ag pool from which they were derived [24]. Human immune IgG also was used to purify a limited pool of proteins from the DOC Ags. As determined by one-dimensional silver-stain analysis, the human IgG used in the present study isolated ~13 DOC-soluble protein bands in the range of 115–25.5 kDa. The human-IgG-Ags induced protective immunity at levels equivalent to that induced by the complete pool of DOC Ags and the immunized mice developed Th2 responses, as indicated by the increase in the eosinophil counts in the diffusion chambers. However, it was of interest to note that...
be the result of a combined immune response simultaneously directed at multiple targets.

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