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Expulsion of Secondary *Trichinella spiralis* Infection in Rats Occurs Independently of Mucosal Mast Cell Release of Mast Cell Protease II

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Our aim was to elucidate the contribution of mucosal mast cells to the effector phase of a secondary immune response to *Trichinella spiralis*. During secondary infection, rats expel 90–99% of *T. spiralis* first-stage larvae from the intestine in a matter of hours. This phenomenon appears to be unique to rats and has been called rapid expulsion. Primary intestinal infection by *T. spiralis* induces mastocytosis, and mast cell degranulation occurs when challenged rats exhibit rapid expulsion. These observations have engendered the view that mast cells mediate rapid expulsion. In this study, we report that immunization of adult Albino Oxford rats by an infection limited to the muscle phase did not induce intestinal mastocytosis, yet such rats exhibited rapid expulsion when challenged orally. Although mastocytosis was absent, the protease unique to mucosal mast cells, rat mast cell protease II (RMCPII), was detected in sera at the time of expulsion. We further evaluated mast cell activity in neonatal rats that display rapid expulsion. Pups born to infected dams displayed rapid expulsion, and RMCPII was detected in their sera. By feeding pups parasite-specific mAbs or polyclonal Abs before challenge infection, it was possible to dissociate mast cell degranulation from parasite expulsion. These results indicate that rapid expulsion can occur in the absence of either intestinal mastocytosis or RMCPII release. Furthermore, release of RMCPII is not sufficient to cause expulsion. The data argue against a role for mast cells in the mechanism underlying the effector phase of protective immunity against *T. spiralis* in rats. *The Journal of Immunology*, 2009, 183: 5816–5822.

**R**odents are natural hosts for *Trichinella spiralis*, a parasitic nematode that colonizes the epithelium of the small intestine. In both mice and rats, primary infection induces a potently Th2 response leading to intestinal mastocytosis (1). Mast cell degranulation can be detected at the time of adult worm expulsion (2–4). The temporal alignment of intestinal mastocytosis and mast cell degranulation with the rejection of adult worms provided evidence that mast cells may be key to intestinal defense against the parasite. Support for this notion has been provided by reports that rejection of both primary and secondary intestinal infections with *T. spiralis* is delayed in mast cell-deficient W/W<sup>−</sup> mice (5, 6) and that the capacity to reject adult worms can be reconstituted with bone marrow cells (7). Studies with wild-type and IL-9 transgenic mice treated with anti-c-kit Abs confirmed these results (8, 9). Furthermore, mice deficient in mast cell protease-1 (mMCP-1)<sup>−/−</sup> show delayed rejection of *T. spiralis* (10). It has been hypothesized that mMCP-1 increases intestinal permeability, providing Abs access to parasite Ags (11); however, the mechanism of adult worm rejection remains unproven.

Rats respond to a secondary *T. spiralis* infection with a dramatic and immediate immune response. This rapid expulsion of first-stage larvae (L<sub>1</sub>) has been likened to intestinal anaphylaxis (12–14). Mice do not exhibit rapid expulsion, although they do reject secondary intestinal infections in an accelerated fashion (15, 16). Mast cell degranulation is coincident with rapid expulsion in rats, as revealed by detection of rat mast cell protease II (RMCPII) in the serum (2); however, there is no direct evidence that mast cells contribute to expulsion. Mast cells have been reported to be the sole cellular source of RMCPII in intestinal tissue of naive rats (17, 18). RMCPII-positive cells are present in the lung, liver, and primary lymphoid tissue (19); however, intestinal mast cells are very likely to be the major source of blood RMCPII, when antigenic challenge is limited to the intestine.

The participation of Abs in rapid expulsion of *T. spiralis* has been documented in passively immunized rat pups. Immune serum or parasite-specific monoclonal IgG1 or IgG2c confers an immunity on pups that is indistinguishable from rapid expulsion displayed by previously infected adult rats (20, 21). Protective Abs are specific for tyvelose-bearing glycans that are unique to L<sub>1</sub> (22). In the presence of tyvelose-specific Abs, L<sub>1</sub> are excluded from the epithelium, entrapped in mucus, or encumbered in epithelial cells (23, 24). Abs are protective when delivered to pups orally or by i.p. injection, or when passively transferred in milk from the mother (20, 21). Passive immunization is not effective in rats after weaning (25). The two rat isotypes known to activate mast cells, IgE and IgG2a, do not protect neonates (21, 26).

In contrast to rat pups, passive immunization of adult rats is effective only after intestinal priming. Infection with either *Nippostrongylus brasiliensis* (27, 28) or *Heligmosomoides polygyrus* (27) enables passively transferred serum Abs or anti-tyvelose
mAbs to confer immunity. Infection with either of these parasites induces mastocytosis as well as many other innate cellular changes in the small intestine. In the studies described in this work, we sought to investigate the requirement for intestinal priming using an immunization scheme that did not involve intestinal infection. In addition, we investigated the activity of mast cells in passively immunized rat pups, because pups do not require intestinal priming for immunity. Our findings indicate that rapid expulsion occurs in the absence of mastocytosis and that mast cell release of RMCPII is neither required nor sufficient for immunity.

Materials and Methods

Rats

Albino Oxford (AO) and PVG strain rats were produced and maintained under specific pathogen-free conditions in the James A. Baker Institute vivarium, according to the guidelines of the American Association for Accreditation of Laboratory Animal Care. Outbred, male NIH-RNU (athymic) and heterozygous NIH-RNU+/+ rats (202N01 Cr:National Institutes of Health-rnu) were obtained from the National Cancer Institute and were barrier maintained. Rats were euthanized by CO₂ inhalation and cervical dislocation. Blood samples were collected from the tails of rats under anesthesia with isoflurane, or from the heart postmortem.

Parasite

T. spiralis (pig strain) was maintained in irradiated adult AO rats, as described by Crum et al. (29). Infectious L₁ were harvested from muscle by digestion in a solution of 1% pepsin-1% HCl at 37°C. Newborn larvae (NBL) were recovered from cultures of adult worms, as described by Beiting et al. (30). Adult rats, 8–12 wk old, were immunized by oral infection with 1000 L₁, or by infection limited to muscle by injection of 80,000 NBL in the lateral tail vein. Adult rats were challenged 9 wk after the immunizing infection. The challenge dose was 500 L₁, for adult rat experiments. Pups (12–16 days old) were challenged with 200 L₁. Small intestines were collected for parasite enumeration 18–24 h postchallenge for adult rats, and 3 h postchallenge for pups.

To evaluate entrapment of intestinal L₁ in mucus, rats were euthanized 60–90 min postchallenge, and the contents of stomach and small intestine (divided into the proximal 50% and distal 50% of the small intestine) were examined, as previously described (24). Briefly, intestinal contents were flushed with 0.85% NaCl, collected into 50-ml polypropylene conical tubes, and packed on ice. L₁ trapped in mucus were observed by pressing mucus between glass slides. L₁ free in saline were considered to have been free in the lumen. Intestines were incubated at 37°C for 5 h, and L₁ migrating out of the epithelium were counted.

Histology

Two-centimeter sections of jejunum were collected, fixed in Carnoy’s solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 3 h, transferred to 70% ethanol, and embedded in paraffin. Mast cells were counted in sections stained with Alcian blue (pH 0.4) and counterstained with Nuclear Fast Red. Goblet cells and eosinophils were counted in sections stained with H&E. Each cell type was counted in 20 crypt-villus units per rat, and values were reported as the mean number per crypt-villus unit for rats in each treatment group.

Sera and Abs

Tyvelose-specific mAbs 9D4 (IgG1), 18H1(IgG2a), and 9E6 (IgG2c) have been described previously (21). Abs were concentrated by (NH₄)₂SO₄ precipitation of mouse ascites fluid, dialyzed against saline, and adjusted to a concentration of 5 mg/ml (21). Immune serum was collected from adult AO rats 9–12 wk following infection with 1000 L₁. Normal rat serum was collected from naive rats. Polyclonal IgE was produced by precipitation of immune or normal serum with 40% (NH₄)₂SO₄, leaving polyclonal IgE in the supernatant (31). Both fractions were dialyzed against saline and concentrated to match the original volume of serum using a 10,000 m.w. cutoff centrifugal device (Millipore-Centricon).

ELISA

Serum Abs were measured using T. spiralis excretory-secretory Ag (32) or anti-IgE (clone A2) (33) for capture, and biotinylated mouse anti-isotype Abs, followed by HRP-conjugated streptavidin. Abs were detected with biotinylated monoclonal mouse anti-rat IgG1 (RB11/39), IgG2a (RG7/11), IgG2b (RG7/11), or IgE (MARE-1). Abs were biotinylated with N-hydroxysuccinimidobiotin (Sigma-Aldrich) in bicarbonate buffer (pH 9.5; 120 μg of biotin per 1 mg of Ab) for 4 h at room temperature and used at 1 μg/ml. Biotin-conjugated anti-rat IgG2c (BD Pharmingen) was used at 0.25 μg/ml. Standard curves for IgG1, IgG2a, IgG2b, and IgG2c used tyvelose-specific mAbs 9D4, 18H1, 10G11, and 9E6, respectively. Sera from rats were diluted 1/6000 for measuring IgG1 and IgG2a, 1:100 for IgG2b, and 1:3000 for IgG2c. To measure total serum IgE, ELISA plates were coated with anti-rat IgE mAb A2 (5 μg/ml), rat myeloma IgE 162 (34) served as a standard, rat sera were diluted 1/100, and biotin-conjugated MARE-1 (5 μg/ml) was used as the detection reagent. RMCPII was detected in rat sera using the RMCPII ELISA kit purchased from Moredun Scientific.

Passive immunization of rat pups

One hour before oral infection with L₁, groups of six to eight rat pups were fed Abs (5 mg/ml in 0.85% NaCl) at a dose of 2.5 mg/20 g body weight. Abs treatments included serum Ig from infected or uninfected rats, tyvelose-specific mAb, and precipitants or supernatants from immune serum. Pups treated with whole serum or serum fractions were fed two doses of 0.5 ml, 2 h and 1 h before challenge infection. Adult rats were passively immunized by i.p. injection of 5 mg of tyvelose-specific mAb or normal serum Ig, and challenged orally 16 h later.

Statistical analyses

Student’s t test was used to compare means among treatment groups. ANOVA and Tukey‘ s least significant difference test were used to detect differences among three or more means. Data are presented as mean ± SD (n = 5–8 rats). In experiments in which pups of infected dams were challenged, means were calculated for each litter and those values assigned to the dam to calculate the mean for the treatment group. For passive immunization experiments, rat pups in each group were selected from a randomized pool from several litters. Differences were considered significant when the p value was less than 0.05.

Results

Immunity induced by i.v. infection is indistinguishable from rapid expulsion

Infection with T. spiralis occurs when a host ingests muscle tissue containing T. spiralis L₁. Infection begins when L₁ invade epithelial cells lining the small intestine, where they molt four times to become adult worms (35). Adult worms in this epithelial syncytium mate, and the females release NBL, which enter the bloodstream and subsequently establish a chronic muscle infection. We have shown previously that rats infected by i.v. injection of NBL develop muscle infections that engender immunity against reinfection of the intestine (36). To confirm that this immunity was equivalent to rapid expulsion induced by natural infection, we evaluated the distribution of larvae in the period immediately following challenge infection. Mucus entrapment of luminal larvae within the first hour of challenge is a hallmark of rapid expulsion (27, 37). Such entrapment occurred in both parenterally and orally infected rats after oral challenge (Fig. 1A). In both groups, L₁ were excluded from the small intestinal epithelium, as evidenced by increased numbers of L₁ in the lumen of the distal intestine (Fig. 1B) and dramatically fewer larvae recoverable from the epithelium (Fig. 1C), compared with naive control animals. Intestinal burdens were not further reduced between 24 and 72 h postchallenge, indicating that the response was specific and restricted to larval, rather than adult, stages (Fig. 1D). The increased number of worms recovered from naive rats at 72 h postchallenge can be explained by the failure of molting larvae (present in the intestine 24 h postinfection) to emerge from the intestinal epithelium. Overall, the kinetics of entrapment and exclusion following intestinal challenge of parenterally infected rats are entirely consistent with rapid expulsion.

Oral challenge of parenterally infected rats causes mast cell degranulation in the absence of mastocytosis

We sought to evaluate the cellular response in the intestine after parenteral infection, including evaluation of mast cell degranulation upon challenge infection. Jejunal mast cells, eosinophils, and...
goblet cells were not increased in parenterally infected rats (Fig. 2, A–C), although there was a mild, but prolonged blood eosinophilia (Fig. 2D). Despite the absence of intestinal mastocytosis, oral challenge of parenterally infected rats induced a significant increase in serum RMCPII concentration (Fig. 2E) that correlated with larval expulsion (Fig. 2F). The amount of RMCPII detected in parenterally immunized rats was consistent with what we have detected in orally immunized rats in several other experiments (1000–2000 ng/ml). These results reveal that degranulation of mucosal mast cells was coincident with expulsion, even in the absence of mastocytosis. Attempts to inhibit mast cell degranulation by treating rats with the mast cell stabilizer, doxantrazole, were unsuccessful. Therefore, we were unable to determine whether mast cell activation contributed to rapid expulsion.

Comparison of Ab responses between naturally and parenterally infected rats revealed a striking difference. Infection limited to the muscle did not cause a significant increase in the total serum concentration of IgE, but did induce a strong parasite-specific IgG2a response (Fig. 2G and H). Both IgE and IgG2a bind to the high-affinity IgE receptor, FcεRI (38), so it is possible that mast cell activation was mediated by IgG2a in parenterally infected rats and by both isotypes in naturally infected rats. Either infection regime induced Ag-specific IgG1 and IgG2c (Fig. 2, H and I), two isotypes known to mediate rapid expulsion in rat pups (21).

Parenteral immunization generates a T cell-dependent mucosal response

Mastocytosis in T. spiralis infection is T cell dependent (39, 40). Because intestinal mastocytosis was not required for rapid expulsion, and knowing that IgG2c mediates rapid expulsion in rat pups and can be produced in a T cell-independent manner (41), we tested the requirement for T cells in rapid expulsion by challenging parenterally infected, T cell-deficient rats. Only euthymic rats demonstrated rapid expulsion, indicating that T cells were required for immunity (Fig. 3). Similar results were obtained in PVG rats made T lymphocyte deficient by thymectomy, followed by irradiation and bone marrow reconstitution (data not shown). In another experiment, we passively immunized parenterally infected rnu/rnu rats with monoclonal, tyvelose-specific IgG1. These animals did not demonstrate rapid expulsion, a result that is consistent with a requirement for T cells in addition to Ab production (data not shown). Blood RMCPII was not elevated in nude rats following challenge, consistent with a failure of both T lymphocytes and T-dependent, Ab production (data not shown).

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**FIGURE 1.** Intestinal immunity induced by muscle infection. A–C, Distribution of larvae in previously infected and naive AO rats 60–90 min postchallenge with 500 L₁. Rats were infected i.v. with 80,000 NBL (muscle) or orally with 1,000 L₁ (natural). Proximal and distal small intestine contents were collected separately, and larvae were counted that were trapped in mucus (A), free in the intestinal lumen (B), or able to migrate into saline from the epithelium (C). D, Intestinal burdens in naive rats and rats immunized by muscle infection. Burdens were stable after 24 h, indicating that immunity was specific for larval stages.

**FIGURE 2.** Cellular and Ab responses to muscle infection. Intestinal sections from rats immunized via muscle infection were evaluated for mastocytosis (A), eosinophilia (B), and goblet cell hyperplasia (C). D, Blood eosinophilia during infection. E, RMCPII concentrations in sera collected 30–90 min postchallenge. F, Intestinal burden 24 h after challenge with 500 L₁ in naive rats and rats infected with muscle infection. G–I, Serum Abs 9 wk following infection with 1,000 L₁ or 80,000 NBL.
Mast cell degranulation is coincident with rapid expulsion in rat pups fostered by infected dams

With the aim of using suckling rat pups to study secondary expulsion of *T. spiralis*, we evaluated mast cell degranulation in this system. Rat pups fostered by a dam infected with *T. spiralis* demonstrated rapid expulsion (20, 42). Passive immunization with tyvelose-specific mAbs confers immunity upon pups born to naïve dams (21). Abs delivered immediately before infection are protective, indicating that cells that may interact with Abs to effect expulsion are resident in the normal neonatal gut. We evaluated mucosal mast cell degranulation in rat pups that displayed rapid expulsion (Fig. 4A). Before challenge, serum RMCPII concentrations were similar in pups born to infected dams compared with pups of naïve dams (Fig. 4B). When both groups were challenged with 200 L1, only pups born to infected dams showed significantly elevated serum RMCPII concentrations (Fig. 4B). Thus, maternal immunity confers upon pups the capacity to activate mast cells upon oral infection with *T. spiralis*.

**RMCPII release does not correlate with rapid expulsion in passively immunized rat pups**

Because the Ab isotypes that provide immunity to rat pups are not the same isotypes known to cause mast cell degranulation in rats, we were able to manipulate this system to separate mast cell degranulation from protective immunity. Pups were fed tyvelose-specific mAbs of different isotypes 1 h before oral challenge (Fig. 5). Treatment with IgG2c afforded protection, but did not cause a significant increase in serum RMCPII concentration. Conversely, IgG2a caused a significant release of RMCPII, but did not provide protection. Pups treated with IgG1 were protected, and modest increases in serum RMCPII were induced. These results indicate that mucosal mast cell degranulation is not required for rapid expulsion. Furthermore, activation of mast cells by IgG2a was not sufficient to cause rapid expulsion.

**RMCPII release is not sufficient to cause rapid expulsion in passively immunized adult rats**

We continued these studies by investigating whether the dissociation of mast cell degranulation from rapid expulsion was possible in adult rats. Five- to 6-wk-old rats (weaned at 3 wk of age) were injected i.p. with tyvelose-specific mAbs 16 h before oral challenge with L1 (Fig. 7). We have previously reported that although treatment with tyvelose-specific IgG2c is not protective in weaned rats, it causes L1 to become encumbered in the intestinal epithelium such that parasites are not released into saline during the standard recovery procedure. This effect is reversed at the time of the first molt (25). In the experiment shown in Fig. 7, we also measured serum RMCPII, and found that the protease was significantly increased in rats treated with IgG2a, but not those treated with IgG2c (Fig. 7B). IgG2a-treated adult
rats did not expel the infection, despite the increase in RMCPII (Fig. 7A). The results confirm that immunity is independent of RMCPII release.

**FIGURE 7.** RMCPII release in passively immunized adult rats. Adult rats were injected i.p. with 5 mg of tyvelose-specific IgG2c or IgG2a, or normal serum Ig (negative control). Intestinal larvae burden (A) and serum RMCPII concentration (B) were determined at 3 and 24 h postchallenge with 500 L1.

**Discussion**

Rapid expulsion is a rare example of an immune response that causes immediate elimination of an intestinal worm. Elucidation of the mechanism of immunity would afford valuable insight applicable to the development of new prophylactic measures that would protect humans and animals against nematode infection. The effort to elucidate the mechanism of immunity in rapid expulsion has been challenged by the combinatorial nature of immune mechanisms in adult rats. Several studies that tested adoptive transfer of lymphocytes or passive immunization with specific Abs failed to fully replicate rapid expulsion in adult recipients (29, 43, 44), suggesting that innate mediators contribute to immunity. In this study, we show that rapid expulsion can be induced by an infection limited to the muscle phase, and that immunity is T lymphocyte dependent. We investigated whether this T-dependent effect involved mucosal mast cells or intestinal mastocytosis.

Previous studies showed a correlation between *T. spiralis* expulsion and mucosal mast cell degranulation, but a specific mediator or causal relationship was not identified (2–4, 45). Rats maintain a resident population of mucosal mast cells in the lamina propria of the small intestine under homeostatic conditions (46). It has been suggested that mast cell activation might be a direct effect of the parasite, and such activation has been demonstrated in vitro following exposure of cultured mast cells to tyvelose-bearing *T. spiralis* Ags (47, 48). In contrast, we found that parasite challenge in the absence of specific Abs did not cause significant RMCPII release. The kinetic of RMCPII release in immune rats was consistent with Ab-dependent activation. In rats displaying rapid expulsion following immunization via muscle infection, IgG2a was the isotype most likely to cause degranulation, whereas in orally infected rats IgE was present in high concentrations. The differences in immune responses induced by intestinal and muscle infection reduce the likelihood of a role for two other innate mediators, goblet cells and eosinophils. Both have been hypothesized to contribute to rapid expulsion.

Throughout the published literature describing rapid expulsion, the most consistent protective effects are attributed to Abs (49). Rapid expulsion displayed by neonatal rat pups born to infected dams can be reproduced in pups born to naive dams by passive immunization with tyvelose-specific IgG (21). The protective mechanisms of the Abs have been documented in vivo (21, 24) and confirmed in an in vitro model (23). Direct effects of Abs on T. spiralis larvae include entrapment of larvae in mucus as well as exclusion from and encumbrance in the epithelium (23, 24). The potential for mast cells to contribute to Ab-mediated protection has not been investigated previously in neonatal rats. Furthermore, the functional activities of mucosal mast cells in neonatal rats had not been tested in the context of infection.

We found that rat pups born to infected dams demonstrated mast cell activation coincident with rapid expulsion. Passive immunization allowed for the separation of RMCPII release from rapid expulsion. It has been established that passive immunization with tyvelose-specific monoclonal IgG1 or IgG2c confers protection on rat pups, whereas monoclonal IgG2a and polyclonal IgE are not protective (20, 21, 26). Thus, the isotypes known to cause mast cell degranulation via FcεRI cross-linking (38, 50), and shown to induce release of RMCPII in our experiments, do not protect pups. Our results show that rat mucosal mast cells are weakly activated by IgG1. IgG1 binds the FcεRI (S. Thrasher and J. Appleton, unpublished observation), although reports of receptor-mediated activation of mast cells by IgG1 are contradictory (38, 50). It is possible that rat mucosal mast cells are activated by IgG1 via binding to other FcR or by some indirect mechanism, such as complement...
activation. Experiments with IgE-rich serum fractions provided confirmation that release of RMCPII was not sufficient to cause rapid expulsion. Conversely, passive immunization with IgG2c was protective, but did not induce RMCPII release, indicating that expulsion of larvae can occur independently of mast cell activation. RMCPII release caused by IgG2a was not sufficient to provide a protective secondary immune response to adult rats. Overall, our results do not support a role for RMCPII or mast cells in rapid expulsion.

Support for the conclusion that mast cells are not pivotal in rapid expulsion was provided by studies using pharmacological agents that block histamine, serotonin, PGs, and intestinal motility (45). Treatment of infected adult rats with inhibitors of various mast cell effectors did not prevent rapid expulsion. The speed of expulsion would not allow for de novo synthesis of mast cell proteins, although cytokines that are prestored by mast cells, such as TNF-α, may play a role (51). Although mMCP-1 promotes adult worm rejection in mice, our results show that release of the equivalent mediator, RMCPII, is not required for rapid expulsion of T. spiralis in rat pups, and that release of RMCPII can occur in pups and adults without causing expulsion.

Infection limited to the muscle phase induced rapid expulsion in the absence of intestinal priming and the cellular changes priming induces. Although this suggests that systemic immunization against a parasitic nematode produced a mucosal immune response, it must be noted that NBL are efficiently delivered to the lung via i.v. injection and some NBL may reach the intestine via the bloodstream. Efforts to inject NBL directly into the muscles of the hind limbs in numbers sufficient to induce immunity consistently allowed a small proportion of NBL to enter the blood and colonize remote sites (data not shown), thus preventing us from determining whether migration through the lung or other tissues was necessary for the induction of immunity. Although we did not detect the obvious cellular changes in the intestines of rats infected by this route, it is possible for stimulation of mucosal associated lymphoid tissue in one site to affect another site. An additional source of cellular mediators may derive from the body cavities in which cellular expansion during muscle infection has been documented in mice (52, 53). Recirculation of cells from the cavities to the intestinal tract, or direct effects of peritoneal responses on the gut have been shown to be a part of the body’s defense against intestinal pathogens (54).

The diaphragm is a preferred site for developing larvae, and the interaction between cavity cells and the parasite in this location may drive subtle alterations in the intestine. Such alterations may differ from those that are commonly associated with nematode infection, such as eosinophilia, goblet cell hyperplasia, or mastocytosis.

Taken together, results from parenteral infection of adult rats and passive immunization of neonates and adults demonstrate that both IgE and IgG2a activate mast cells during rapid expulsion. Our data support the conclusion that neither mastocytosis nor release of RMCPII is required for rapid expulsion. Identification of the factor that enables Abs to protect adult rats requires further investigation. Our results have significance for the development of vaccines against helminths, because they show that protective immunity can be engendered without inducing a potentially dangerous allergic response to Ag.

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Disclosures
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