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Cercarial Dermatitis Caused by Bird Schistosomes Comprises Both Immediate and Late Phase Cutaneous Hypersensitivity Reactions

Pavlína Kouřilová, Karen G. Hogg, Libuše Kolářová, and Adrian P. Mountford

Avian schistosomes are the primary causative agent of cercarial dermatitis in humans, but despite its worldwide occurrence, little is known of the immune mechanism of this disease. Using a murine model, hosts were exposed to primary (1×) and multiple (4×) infections of Trichobilharzia regenti via the pinna. Penetration of larvae into the skin evoked immediate edema, thickening of the exposure site, and an influx of leukocytes, including neutrophils, macrophages, CD4+ lymphocytes, and mast cells. A large proportion of the latter were in the process of degranulating. After 1× infection, inflammation was accompanied by the release of IL-1ß, IL-6, and IL-12p40. In contrast, in 4× reinfected animals the production of histamine, IL-4, and IL-10 was dramatically elevated within 1 h of infection. Analysis of Ag-stimulated lymphocytes from the skin-draining lymph nodes revealed that cells from 1× infected mice produced a mixed Th1/Th2 cytokine response, including abundant IFN-ß, whereas cells from 4× reinfected mice were Th2 polarized, dominated by IL-4 and IL-5. Serum Abs confirmed this polarization, with elevated levels of IgG1 and IgE after multiple infections. Infection with radiolabeled cercariae revealed that almost 90% of larvae remained in the skin, and the majority died within 8 days after infection, although parasites were cleared more rapidly in 4× reinfected mice. Our results are the first demonstration that cercarial dermatitis, caused by bird schistosomes, is characterized by an early type I hypersensitivity reaction and a late phase of cutaneous inflammation, both associated with a polarized Th2-type acquired immune response. The Journal of Immunology, 2004, 172: 3766–3774.

Cercarial dermatitis (or swimmer’s itch) is a severe inflammatory reaction caused by penetration of the skin by schistosome parasites, frequently of the genus Trichobilharzia (1). Species of these bird schistosomes conventionally parasitize ducks, but they can also invade mammals as nonspecific hosts, resulting in inflammation of the skin at the site of infection (1). The disease develops after repeated contact with infectious cercariae and is of increasing importance in human populations throughout large parts of Europe and America (1–3). However, due to the lack of a specific diagnostic test, the total number of cases is unknown, but it is likely to be vastly underestimated due to under-reporting and misdiagnosis. Cercarial dermatitis is now considered an important emerging disease to be vastly underestimated due to under-reporting and misdiagnosis.

Penetration of vertebrate skin is the key point in the parasite life cycle. Cercariae must recognize and invade the skin, where they have to adapt to the host environment, resulting in metabolic and morphological changes that aid migration and immune evasion. Once in the avian host, the parasite may continue to migrate to other tissues, but the precise route is not fully understood. The recently described Trichobilharzia regenti migrates via peripheral nerves to the CNS, and as a consequence, it causes serious neuromotor disorders (e.g., balance and orientation disorders, leg paresis) (4, 5). In ducks, it ultimately matures in the nasal tissues (4). Schistosomula of the visceral species Trichobilharzia szidati (Syn T. ocellata) (6) probably enter lymphatic or venous vessels in the skin and continue via the lungs and systemic circulation to the hepatic-portal system as the final location (1, 6, 7). Both T. regenti and T. szidati can also infect the mammalian host via a percutaneous route, but neither successfully matures. However, some larvae can reach the lungs (T. szidati) (7) or the spinal cord and brain (T. regenti), where they cause pulmonary and neuromotor disorders, respectively (4, 8). The fate of nonmaturing larvae in mammals is not fully known, although we suggest that most of them die in the skin (9).

The death of larvae in the skin and/or the release of abundant proteases may explain why cercarial dermatitis to Trichobilharzia is such a frequent pathological condition in mammals, particularly after reinfection (10–13). Although cercarial dermatitis is frequently referred to as type I allergic hypersensitivity reaction, there is a complete lack of contemporary studies to support this hypothesis. Histopathological observations show that infection by T. regenti leads to the rapid development of an acute inflammatory reaction in the skin (14) characteristic of severe edematous cercarial dermatitis in humans (15). In this study we show in a murine model of T. regenti infection that the immune response in the skin has the hallmarks of an immediate hypersensitivity response, followed by a late phase of inflammation. In addition, the acquired immune response, as judged by lymphocyte reactivity in the skin-draining lymph nodes.

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nodes (sdLN)\(^3\) and serum Abs, is highly biased toward the Th2 pole in reinjected mice. Finally, we describe the pattern of \(T.\ regenti\) larval migration after infection of naive and reinjected skin and report that few parasites exit from the site of exposure, particularly in reinjected hosts, and the few that do so enter the CNS.

Materials and Methods

Parasites and parasite Ag preparations

\(T.\ regenti\) was routinely maintained in Radix peregra snails, as intermediate hosts, and in Anas platyrhynchos L. dom. ducks, as final hosts (Charles University, Prague). \(T.\ regenti\) cercariae were induced to shed by exposure of infected snails to bright light, and then the pinnae of C57BL/6 mice (female, 8–12 wk old; University of York or Charles University) were exposed to cercariae in the dark for 15 min as described previously for Schistosoma mansoni (16, 17). After counting nonpenetrant cercariae, the infection dose (i.e., those that successfully penetrated) was calculated to be between 230 and 300 parasites/pinna. Mice were reinjected with 150–200 cercariae on the same sites 10, 20, and 30 days later. On day 30, a separate group of age-matched naive mice was infected (1×) alongside reinjected animals (4×). One group of reinjected mice was not re-exposed to cercariae for a fourth time on day 30 and served as a control group (RC).

To yield Ag preparations for lymphocyte stimulation, pools of cercariae were concentrated over ice, resuspended in a minimal volume of PBS, and then frozen at −20°C. Several aliquots of cercariae prepared on different days were then pooled, sonicated, and centrifuged at 100,000 \(\times\) g for 1 h at 4°C. The resultant soluble preparation (\(T.\ regenti\) Ag) was assayed for protein content using Coomassie Plus-200 protein assay reagent (Pierce, Rockford, IL) and sterilized by exposure to UV light.

Infection with radiolabeled parasites

To determine the number of parasites staying in the skin after the primary and repeated infections, mice were infected with either 30 radiolabeled cercariae of \(T.\ regenti\) via each pinna and killed on days 1, 2, 3, 5, and 8 with or 300 cercariae via the shaved thigh and killed on days 3 and 7. In the case of reinfection, mice were infected times times with normal parasites on the same site; the fourth infection used radiolabeled cercariae.

To label \(T.\ regenti\) 60 snails with a patent infection were placed in 30 ml of water containing \[^{35}\text{S}\]methionine and cysteine (Pro-mixL-[\(^{35}\text{S}\]) in C and placed in contact with phosphor storage screens for overnight at 60°C. Remaining bone tissue, including skull, spine, and leg bones, was squashed and placed on cards, covered with plastic cord, brain, lungs, liver, spleen, sdLN, kidney, and heart) were removed, ed times, and the soft tissues (skin, nerves, spinal cariae were labeled. After the infection procedure, the number of nonpen-cercariae obtained from the labeled snails 5 days later when 100% of cer-cercariae were induced to shed by

Experimental regimen for determination of immunological readouts

Serum samples were obtained after collection of peripheral blood from the tail on days 10, 20, and 30, just before each reinfection. To determine the extent of inflammation, pinna thickness was measured before each infection and at 30 min (0.5 h) and 1 h after infection using a dial gauge micrometer (Mitutoyo, Andover, U.K.). At various time points after infection on day 30 (0.5, 1, 6, and 18 h, and days 2, 4, and 8), mice from the 1× and 4× groups were killed, and their pinnae and sdLN were removed and prepared for in vitro culture or histological analysis.

Histology

For histological analysis, pinnae were fixed in 10% neutral buffered formalin (Sigma-Aldrich, Poole, U.K.), wax-embedded, sectioned at 6 μm, and stained with H&E, Alcian Blue/Safranin, or Toluidine Blue (Faculty Hospital, Bulovka, Czech Republic; or Eastalt Histology Laboratories, Warrington, U.K.). Mast cells were identified by red coloration after Alcian Blue/Safranin staining; their number across the pinna (between the dorsal and ventral surfaces) was determined along 30 representative 500-μm fields. The proportion of degranulating mast cells among the total mast cell population was determined by visual examination of 200 mast cells/time point ×1000 magnification. For immunohistochemistry, pinnae were frozen in liquid N\(_2\) and stored at −80°C. Sections (10 μm) were fixed with 3.6% paraformaldehyde and prepared as described previously (16). Endog-enous peroxidase and avidin/biotin were blocked with 1% H\(_2\)O\(_2\)-PBS and an avidin/biotin blocking kit (Vector Laboratories, Peterborough, U.K.). Cell types were identified using biotinylated or unlabeled Abs against various surface markers as follows: MHC II \(^\text{1}\) APCs (I-A\(^\text{b}\); Caltag MedSys-tems, Towcester, U.K.), macrophages (F4/80; Serotec, Oxford, U.K.), granulocytes (Gr-1; BD PharMingen, Oxford, U.K.), neutrophils (7/4; Se-rotec), and Th lymphocytes (CD4; Caltag MedSysytems). Specific Abs or the appropriate isotype controls were applied to the tissue for 45 min at room temperature and then probed, where required, with biotinylated sec-ondary Abs. Positive staining was revealed using Vectastain Elite ABC peroxidase complex combined with Vector VIP enzyme substrate and counterstaining with methyl green (Vector Laboratories).

In vitro culture of sdLN

Skin biopsies were cultured in vitro as described previously (16, 17). Briefly, pinnae were sterilized in 70% ethanol, split into two faces, and floated onto 1 ml of RPMI 1640 medium containing 10% FCS (low endotoxin; Seralab, Oxon, U.K.), 2 mM l-glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich; RPMI 1640/10) in 24-well hydrophobic culture plates (Greiner Laborotechnik, Frickenhausen, Germany). Biopsies were cultured at 37°C in 5% CO\(_2\). After 2 and 18 h, 0.5 ml of the supernatant was collected and then frozen at −20°C before analysis of the histamine and cytokine content.

In vitro culture of sdLN

Single-cell suspensions of sdLN were prepared and cultured (4 × 10\(^5\) cells/well) at 37°C in 5% CO\(_2\) in 96-well plates. Cells were stimulated with or without plate-bound anti-CD3 Ab (5 μg/ml; BD PharMingen) or Tr Ag (50 μg/ml). Culture supernatants were removed 24 and 48 h after anti-CD3 or Tr Ag stimulation, respectively, and frozen at −20°C before cytokine determination. Cell proliferation was measured by incorporation of \[^{3}H\]thymidine (0.5 μCi/well; Amersham Pharmacia Biotech, Little Chal-font, U.K.) after an additional 18-h culture. Cells were harvested and then processed for scintillation counting (TopCount; Packard, Pangbourne, U.K.).

Cytokine and histamine production

Paired Ab capture ELISAs were used to measure the cytokines IL-1β, IL-4, IL-6, IL-10, IL-12p40, and IFN-γ in the 2 and 18 h dermal supernatants as described previously (16). For sdLN cell culture supernatants, capture ELISAs were used to detect IFN-γ, IL-4, IL-12p40, and IL-10 (16), and IL-5 (17). A histamine competitive ELISA kit (IBL, Hamburg, Germany) was used to detect the detection of histamine production in skin cell culture supernatants (2-h culture) according to the manufacturer’s instruction. Re- actions were detected using tetramethylbenzidine substrate (Kierkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was read at 630 nm using a plate reader (MRX II; Dynex Technologies, Billinghamurst, U.K.).

Ab response

Total Ag-specific IgG, IgG1, IgG2a, and IgG2b Ab responses to Tr Ag were measured by ELISA. Briefly, Immuno plates (Nunc. Naperville, IL) were coated with 0.5 μg/ml Ag in carbonate coating buffer (pH 9.6) and left at 4°C overnight (18). Plates were probed with serial serum dilutions and then with peroxidase-conjugated anti-mouse IgG, IgG1, IgG2a, and IgG2b Abs (Zymed Laboratories, San Francisco, CA) diluted 1/1000. Total Ab was determined by capture ELISA, and binding levels were compared relative to a standard curve of recombinant IgE (17). Binding reactions were visualized using tetramethylbenzidine substrate and read at 630 nm using a plate reader (MRX II; Dynex Laboratories).

Statistics

Statistical analyses were performed using Student’s t test. Values of \(p < 0.001, p < 0.01,\) and \(p < 0.05\) were considered significant. Data are the mean of a minimum of four to six samples per time point. The experiments shown are representative of two or three repeats. Spearman’s coefficient of rank correlation was used to compare the number of degranulating mast cells at times postexposure with the amount of histamine detected.

\(^3\) Abbreviations used in this paper: sdLN, skin-draining lymph node; p.i., postinfection; RC, reinfection control; Tr, soluble cercarial antigen from Trichobilharzia re- genti; 1×, primary infection; 4×, multiple infections.
Results

Penetration of cercariae to skin rapidly evokes edema and an influx of inflammatory cells

Penetration of the skin by *T. regenti* cercariae had substantial effects on inflammatory reactions at this site. We recorded an immediate increase in pinnae thickness at 0.5 and 1 h after the first exposure (*p* < 0.001; Fig. 1). Inflammation continued to increase slowly over the ensuing 8 days, but had declined again by day 10 (time of second infection). After each successive reinfection, we noted an immediate increase in pinna thickness within 0.5–1 h, which was particularly evident after the third and fourth reinfections (*p* < 0.001). After the fourth reinfection, the pinna thickness at peak values was 4.4-fold greater than that in naive mice. The considerable thickening of the skin 1 h after the last challenge (+57.5%) decreased by 18 h postinfection (*p* < 0.01) and then remained at approximately the same level up to at least day 8, when inflammation was still 3.2-fold greater than that in naive mice.

Although some shrinkage occurred after sample preparation, making direct comparison with pinnae thickness (see Fig. 1) difficult, histological examination confirmed the profile of inflammation (Fig. 2). In 1× infected mice, larvae penetrated the stratum corneum within 1 h and evoked an influx of cells by 6 h p.i. By 18–48 h, most parasites were observed within the epidermis or upper layers of the dermis, surrounded by large inflammatory cellular foci. However, by days 4–8, the cellular infiltrate appeared to be sloughed off, with the underlying epidermis showing signs of hyperkeratosis. In the 4× reinfected skin, strong perivascularitis, folliculitis, and cellular influx dispersed throughout the entire dermis were noted. Moreover, the presence of extensive inflammatory foci resulted in the formation of large abscesses and subsequently in the development of dermal and epidermal necrosis, usually by day 4 p.i. Intraepidermal pusulae and parakeratosis were abundant features at sites of previous parasite penetration, and similar to 1× infected mice, areas of tissue sloughing were observed to overlay regions of tissue repair or regeneration. Parasite residues within cellular infiltrates were in some cases detectable in the dermis up to day 8 p.i.

More detailed immunohistochemical analysis of the dermal site of infection on day 2 after 1× or 4× infection revealed the heterogeneous nature of the cellular influx (Fig. 3). Gr-1^+^ granulocytes and 7/4^+^ neutrophils were highly abundant by day 2 in 1× infected mice and were scattered in groups throughout the dermis. Also present were significant influxes of MHC II^+^ APCs and F4/80^+^ macrophages, also in patches throughout the dermis. However, only a small number of CD4^+^ lymphocytes were detected in 1× infected skin by day 2. The infiltration of each cell type was substantially elevated in 4× reinfected mice by day 2. Indeed, the influx of granulocytes/neutrophils was so great it was difficult to distinguish individual cells. MHC II^+^ and F4/80^+^ cells were also highly abundant after reinfection and were distributed throughout the dermis, but at lower densities than for granulocytes. Finally, the number of CD4^+^ was substantially increased in 4× reinfected mice. Visual observation of erythema in intact pinnae was most evident between 1 and 18 h after the fourth reinfection (data not shown).

**Th2-promoting cytokines dominate in skin after repeated infections**

Various cytokines were detected in supernatants collected after 2- and 18-h in vitro culture of skin biopsies from 1× and 4× infected mice (Fig. 4). IL-1β, IL-6, IL-4, and IL-10 were all detected at 2 h (Fig. 4, a, c, f, and h), but were more abundant at 18 h (Fig. 4, b, d, g, and i), whereas IL-12p40 and IFN-γ were only detected at 18 h (Fig. 4, e and j). The production of both proinflammatory...
IL-1β and IL-6 by skin biopsies from 1× infected mice peaked soon after infection before declining to naive levels by days 4–8 (Fig. 4, a–d). Greater levels of IL-1β were detected in the culture supernatants obtained from 4× reinfected mice, although levels had returned to near naive levels by days 4 and 8 p.i. (Fig. 4, a and b). However, the production of IL-6 in the skin of 4× reinfected mice was not substantially different from that seen in 1× infected mice, although the peak of production was detected slightly earlier (p < 0.001, 6 vs 18 h; Fig. 4, c and d).

Only limited quantities of IL-12p40 were detected immediately after infection in 1× infected mice, but this increased progressively above naive levels to a peak (3.5-fold) on day 8 p.i. (p < 0.001; Fig. 4e). High levels of IL-12p40 were also detected in skin biopsies obtained from RC mice immediately before the fourth infection (p < 0.001 vs naive), but a dramatic decrease in the quantities detected was evident within 1 h after reinfection (p < 0.001). Thereafter, the quantities of IL-12p40 present in the culture supernatants increased to a peak on day 8, similar to levels in 1× infected mice. Indeed, the kinetics of IL-12p40 production in 1× infected mice peaked at 205.1 ng/ml, which continued progressively up to day 8 p.i. to a level of 125.1 ± 21.3 ng/ml. The level of histamine production in RC mice just before reinfection was still elevated over that in naive mice (p < 0.01). However, 1 h after reinfection, histamine reached a peak of 205 ± 47.4 ng/ml (p < 0.05). After this point, there was a slight decline over the ensuing 8 days, but it was still elevated compared with levels in naive and RC mice.

Cytokines associated with Th2-type immune responses were scarce in culture supernatants from 1× infected mice, but were markedly increased immediately after reinfection (Fig. 4, f–i). Very low levels of IL-4 were detected in 1× infected skin on days 4 and 8 p.i. only (p < 0.05 to p < 0.01; Fig. 4g). Within 1 h of reinfection, there was a massive 7-fold increase in the amount of IL-4 secreted by skin from 4× reinfected mice compared with biopsies taken from RC mice just before reinfection (Fig. 4g; p < 0.001). In the 4× reinfected group, abundant IL-4 continued to be detected for biopsies taken between 1 h and 2 days (p < 0.01), but the levels decreased dramatically by days 4 and 8 p.i. IL-10 was expressed at low levels in 1× infected mice at all time points (Fig. 4, h and i). However, in RC mice the amount of IL-10 had increased, and after the fourth infection, a surge in production (3.5-fold) was recorded within 1–6 h (Fig. 4, h and i). As for IL-4, the highly elevated levels of IL-10 were maintained in biopsies taken between 1 h and 2 days, after which there was a marked decline to levels similar to those seen in RC mice, but still significantly above the levels in naive mice. In comparison, only small quantities of IFN-γ were detected (sdLN cells; see Fig. 6), and these tended to be produced at later times p.i. (i.e., days 2–8; Fig. 4j).

**FIGURE 3.** Immunohistochemical analysis of cellular infiltrate into the skin after exposure to T. regenti. Transverse cryosections of pinnae obtained on day 2 after 1× infection and 4× infection were stained for neutrophils (7/4), granulocytes (Gr-1), lymphocytes (CD4), APCs (MHCI), and macrophages (F4/80). There was minimal staining for any marker on naive mice (example shown for 7/4) or by isotype control mAb (shown for IgG2a control on 4× reinfected skin). Scale bar = 100 μm.
when the total number of mast cells per field was increased ~5.7-fold compared with that in naive mice (Fig. 5e). The staining protocol also identified mast cells in the process of degranulation (see Fig. 5d). The number of mast cells in the process of degranulation vs intact mast cells was greater in both 1X and 4X infected mice compared with naive mice (Fig. 5e). The number of degranulating mast cells was also greater in 4X reinfected than in 1X infected animals. Furthermore, the amount of histamine detected at various times after exposure was closely related to the number of degranulating mast cells (r = 0.832; p < 0.01).

Reinfeciton causes a shift toward Th2-associated cytokine production in sLN

Cells from sLN (draining the pinnae) obtained from 1X infected and 4X reinfected mice 4 and 8 days after infection were compared for the ability to proliferate after stimulation with soluble Tr Ag or plate-bound anti-CD3 Ab. There was little difference in the extent of proliferation between the two groups of mice at either time point in response to anti-CD3 (data not shown). However, in response to parasite Ag, cells from 1X infected mice on both days 4 and 8 incorporated greater quantities of [3H]thymidine than cells from 4X infected mice (p < 0.001; Fig. 6a). Cells from 1X infected mice secreted high levels of IFN-γ on days 4 and 8, but IFN-γ was hardly detected or its production was significantly lower using cells from 4X infected mice (Fig. 6b; decreased by 140-fold on day 4 (p < 0.001) and 15-fold on day 8 (p < 0.01)). In contrast, there was little difference in the absolute quantities of IL-4 and IL-10 detected between 1X and 4X infected mice on days 4 or 8, but for both groups of mice, significantly more cytokine was detected at the earlier time point (p < 0.001). IL-5 was also detected in both 1X and 4X infected mice, but on day 8 p.i. the quantity produced in the 4X reinfected group was much greater than that in the 1X infected group. Taking all cytokine results for the sLN together, IFN-γ was one of the most abundant cytokines in 1X infected mice. However, a combination of lower IFN-γ and increased IL-5 in 4X reinfected mice suggests that these mice have a more Th2-like immune response than 1X infected mice.

Serum Ab isotypes show a Th2-type bias in reinfected mice

The IgG Ab response to Tr Ag and total IgE was analyzed in serum samples collected before each successive infection and 8 days after the last challenge (Fig. 7). The levels of Ag-specific Th1-associated IgG2a increased marginally over the background values to a peak on day 8 after the fourth infection (p < 0.05), whereas IgG2b hardly increased at any time point. In contrast, there was a marked increase in the Th2-associated IgG1 isotype particularly after the third and fourth infections. Indeed, by day 8 after the fourth infection, the OD values were 4.5-fold greater than those in naive mice (p < 0.01). The total serum IgE levels were significantly elevated after the second infection and each successive reinfection (p < 0.01 to p < 0.001). The amount of IgE reached a 68-fold increase 8 days after the fourth reinfection compared with naive mice.
Most T. regenti larvae die in skin

To establish the fate of invading larvae, infection of the skin was performed with radiolabeled parasites. Using a low dose of infecting (1×) cercariae (30/pinna), a rapid, but progressive, decline in the number of parasites was detected in 1× infected mice, with <10% remaining by day 8 (Fig. 8a). Nearly all parasites were present in the skin tissue, with the exception of one mouse in which, on day 2, two were found in the lungs and one in the brain (data not shown). To more accurately establish the tissue distribution of parasites and to compare the locations of larvae in 1× and 4× infected mice, experiments were performed using a higher infection dose (300 parasites/mouse). In 1× infected animals, 58.8 and 22.3% of the penetrating parasites were subsequently detected on days 3 and 7, respectively. A very large proportion of the parasites detected stayed in the skin (87.6% on day 3 and 94.6% on day 7).

FIGURE 5. Immediate production of histamine and mastocytosis is a feature of infected skin. a. Histamine production by in vitro-cultured pinnae obtained from 1× infected and 4× reinfected mice at times after exposure. Supernatants were obtained in the absence of added Ag after 2 h in vitro culture. Bars represent histamine production in nanograms per milliliter, equivalent to the total per pinna. Data are the mean ± SEM for four to six samples. Statistical significance is shown for the 1× infected group vs naive and for the 4× reinfected group vs RC. The horizontal dashed line shows histamine production in naive mice. b–d. Detection of mast cells in the exposure site illustrated by representative transverse sections through pinnae stained with Alcian Blue/Safranin. b. Naive pinna. c. Pinna at 18 h from 4× reinfected mice; mast cells are indicated by arrows. d. High power image shows a mast cell in the process of degranulation. e. Numbers of mast cells per field (×400 magnification) in transverse sections of pinnae at times after 1× and 4× infection. f. Numbers of intact mast cells, g. Numbers of degranulating mast cells at different time points. Values are the mean ± SEM. Statistical significance is shown for the 1× infected group vs naive (day 0) and for the 4× reinfected group vs RC values.

The majority of the remaining parasites migrated to the peripheral nerves and CNS (spinal cord and medulla oblongata; 11.2% on day 3 and 5.3% on day 7), but <1.2% was detected in the lungs and slDN (Fig. 8b). The decline in the number of larvae detected was faster in 4× reinfected animals. In total, only 32.4 and 14.3% of penetrating parasites were detected on days 3 and 7, respectively. Moreover, very few parasites were able to escape from the skin and migrate normally to the CNS. However, in comparison with 1× infected mice, a greater number of parasites detected after the fourth infection was localized in slDN (8.5% on day 3 and 18.8% on day 7).

Discussion

Historically, cercarial dermatitis has been described as an immediate hypersensitivity or allergic reaction (3, 9, 15, 19), but there are few recent histopathological reports to support this hypothesis. For the first time we present immunological data showing that cercarial dermatitis comprises immediate hypersensitivity, followed by a late phase inflammatory reaction, and we identify the parasites’ death in the skin as the main causative agent.

Exposure of mice to T. regenti results in an inflammatory reaction remarkably similar to that recorded for skin biopsies taken from human patients (1, 9, 14, 15); therefore, we believe that the mouse is a good model to examine the immunological mechanism. Skin becomes inflamed within 30 min of 1× exposure to cercariae. After each subsequent infection, substantial inflammation occurs within 1 h, with dramatic increases seen after the third and fourth infections, indicative of an immediate type I reaction. Moreover, the pinnae at early times are edematous, leaking copious fluid during tissue preparation. This is accompanied by visible, but transient, erythema resulting from vasodilation, which is also a feature of the clinical condition.

A major mediator of local vascular permeability, and consequently edema, is histamine produced by activated mast cells and basophils (20). In this context, we observed elevated histamine production by the skin within 1 h of T. regenti infection, which correlated with an increase in the number of degranulating mast
cells. Tissue mast cells are the principle effector cells in immediate-type, Th2-associated allergic reactions (21), but they also play an important role in innate immune responses (22). Histamine is preformed and stored in secretory granules; therefore, it can be released immediately after stimulation, as shown in the in vitro culture supernatants of skin biopsies after only 2 h. The production of histamine in 1X infected mice occurred in the absence of parasite-induced Ab response; therefore, we infer that mast cells, in response to a primary infection with *Trichobilharzia*, are activated and degranulate in an IgE-independent mechanism. Indeed, cercariae from *S. mansoni* were able to initiate histamine release from rat peritoneal mast cells in the absence of IgE (23). However, mast cells can be activated by bacterial LPS (24), so we cannot exclude the possibility that bacterial contamination of parasite-infected skin contributes to triggering of the initial dermal response. Nevertheless, the quantity of histamine produced by skin biopsies was substantially greater in 4X compared with 1X infected mice. This was mirrored by an increase in the number of mast cells in the skin and elevated levels of serum IgE in the reinfected group. Very high levels of IL-4 were also detected in the supernatants of skin biopsies from 4X, but not 1X, infected mice. As IL-4 was dramatically up-regulated within 30 min of reinfection, we conclude that this cytokine must also be released from preformed granules in mast cells, rather than as a newly synthesized product from T cells. Conventionally, it is thought that mast cells degranulate, thereby releasing histamine and IL-4, after the binding of IgE aggregated with multivalent or bivalent Ag via high affinity FceRI on their surface (21). Indeed, with time after infection we observed an increasing number of mast cells in the pinnae in the process of degranulation. Therefore, it is logical to conclude that the production of histamine and IL-4 immediately after the fourth reinfection in the presence of abundant IgE in our infection model occurs via IgE-dependent mast cell degranulation. We also predict that the immediate inflammatory response is likely to be at least in part systemic in operation. However, it would be instructive to identify differences in the timing and composition of the inflammatory response that may occur between local and distant skin sites after reinfection.

Although our data provide good evidence that *Trichobilharzia* initiates a classic immediate hypersensitivity reaction, the pinna thickness in 1X infected mice continues to increase from 1 h up to day 8. Moreover, although the immediate increase in pinna thickness in 4X reinfected mice subsides within 24 h after parasite exposure, the pinnae remain inflamed over the following week relative to naive levels. This suggests that there is a late phase of inflammation, possibly caused by sustained/high doses of allergen (20), as would be produced by the presence of parasites in the skin. Although IgE-dependent histamine release from mast cells can contribute to the induction of the late phase in skin (25, 26), the dermal inflammatory response during the late phase (18 h to 2 days) conventionally comprises a heterogeneous population of cells, with mast cells representing only a minor component. Indeed, our immunohistochemical studies demonstrate the presence of numerous granulocytes (including neutrophils) and macrophages in the skin of both 1X and 4X *T. regenti*-infected mice, both of which will release soluble mediators involved in the dermal response. It is generally agreed that the late phase inflammatory reaction between 12 and 48 h is due to leukocytes recruited to the tissue rather than mediators released by mast cells, and that the late phase reaction is closely associated with the release of IL-4 and IL-13 (i.e., is strongly Th2-associated) (20, 27). In this context, skin-derived IL-4 was abundant particularly in 4X reinfected mice over the period when the late phase reaction was greatest. There
was also an influx of CD4+ cells into the skin at this time, indicating that they could be a source of IL-4. Inflammation at later times (days 4–8) may be a continuation of the allergic late phase reaction and/or may involve delayed-type responses traditionally thought to be Th1-associated, involving IL-12 and IFN-γ, which we show are more abundant at later times.

The immediate phase of inflammation in reinjected skin is characterized by a rapid increase in the secretion of IL-4 and IL-10 and an immediate suppression of proinflammatory IL-12p40. The release of IL-12 (and limited quantities of IFN-γ) re-emerges with time as the levels of IL-4 and IL-10 subside. This supports the hypothesis that the production of IL-12 in the skin is closely regulated by IL-4 and/or IL-10. Indeed, after exposure to S. mansoni, IL-4R-deficient (17) and IL-10-deficient (28) mice had elevated levels of skin-derived IL-12p40. In the current study IL-12p40 secretion is greatest during the later phases of inflammation, when the skin is undergoing renewal and resolution of the epidermal microabscesses, and recently it has been suggested that IL-12 promotes tissue repair of skin cells by enhancing DNA repair (29). The relative abundance of IL-12 in 1× infected mice in the absence of IL-4 and IL-10 also correlates with greater Th1-associated IFN-γ production by cells from sdLN.

In contrast, the development of cercarial dermatitis in 4× reinjected mice correlates with the dominance of Th2-type cytokines released from pinnae and Ag-stimulated cultures of cells from sdLN. One of the first and most abundant cytokines to be detected is IL-6, which can guide Th2-type polarization via the induction of IL-12p40. Indeed, after exposure in 4× compared with 1× infected mice, suggesting that prior sensitization leads to a quicker response. Although we have not been able to define the cellular sources of IL-4 and IL-10, both can derive from Ag-stimulated Th2 cells and mast cells. IL-4 has an enhancing effect on the proliferation and mediator release by mast cells, thereby providing positive feedback (32). IL-10 will also promote Th2-type responses, but can act as a powerful regulator of Th cell activity (33), thus providing a possible explanation for the lower levels of cell proliferation in the sdLN of 4× infected mice. IL-10 is in addition an important regulator of schistosome-induced dermal inflammation (28). In this context, both T. ocellata and S. mansoni cercariae release similar types and quantities of eicosanoids (34), which are major factors in the regulation of IL-10 production by skin-derived cells (35).

Finally, it is known that histamine has a major role in priming for Th2-type responses and IL-10 production through its ability to bind to the H2 receptor (H2R) (36–38). Binding of H2R on monocytes and dendritic cells leads to inhibition of IL-12 production, but enhanced IL-10, causing these cells to drive CD4+ cells toward the Th2 phenotype (39, 40). Furthermore, Th2 cells predominately express H2R, whereas Th1 cells express H1R, ligation of which results in IFN-γ production (41). Consequently, in an environment rich in Th2-type cells, such as the pinnae of 4× infected mice, binding of histamine would push further to the Th2 pole and favor IL-4-mediated isotype switching to Th2-associated IgG1 and IgE. As such, we recorded increases in IgG1 and IgE after the third and fourth reinfections, but there was little change in the level of IgG2a.

The inflammatory events noted above are directly caused by penetration of the skin by T. regenti larvae. Our parasite-tracking data demonstrate that the vast majority of larvae in 1× infected mice do not migrate beyond the skin, and nearly all die at this site by day 8. Indeed, by day 3, between 40 and 60% were not detectable, which contrasts with a parallel experiment in which >80% of labeled S. mansoni larvae migrated from the pinnae and were still detectable by day 8 (data not shown). It is probable that proteases released by the T. regenti larvae (42) in the epidermis during the first few hours to aid skin penetration are the major cause of the immediate inflammatory response, as secreted material from S. mansoni cercariae with proteolytic activity are potent stimulators of mast cell activity (43). Proteases are good allergens (44), and modeling studies show that certain Schistosoma proteases share IgE epitopes with the allergen Der P-1 from house dust mites (45). However, the early death of so many T. regenti larvae in the skin, which is even quicker after reinfection, provides a plentiful supply of Ag to cause a late phase reaction even when only a few parasites remained by day 8. T. regenti conventionally exhibits a neurotropic mode of migration to reach the brain of both avian and mammalian hosts (4, 5), but the immune response after the fourth infection significantly reduced the proportion of larvae that entered neural tissues by day 3 (11.3% in 1× infected mice vs <1% in 4× reinjected mice). The enhanced death of larvae in 4× reinjected skin most likely results from the much greater inflammatory reaction in the skin of these animals and is largely host-protective against onward parasite migration, albeit at the cost of severe dermal inflammation.

Together our observations provide evidence for the first time that cercarial dermatitis in the mammalian host is initially a type I, immediate hypersensitivity response, followed by a late phase reaction induced by the presence of T. regenti larvae in the skin. The immune response is clearly a Th2-associated phenomenon. Our observations raise thoughts about the nature of immune responses to repeated infection with mammalian Schistosoma, which also might favor the development of Th2-type responses. This has relevance, because protective immunity to schistosomes in humans has been linked with Th2-associated responses. We are now in a position to further dissect the immunological basis of cercarial dermatitis with a view to developing therapeutic strategies to treat this condition and other parasite-induced allergic reactions of the skin.

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References


