Immunohistochemical Investigations of Treatment with Ro 13-3978, Praziquantel, Oxamniquine, and Mefloquine in Schistosoma mansoni-Infected Mice

Gordana Panic, Marie-Thérèse Ruf, Jennifer Keiser
Swiss Tropical and Public Health Institute, Basel, Switzerland, and University of Basel, Basel, Switzerland

ABSTRACT
To date, there is only one drug in use, praziquantel, to treat more than 250 million people afflicted with schistosomiasis, a debilitating parasitic disease. The aryl hydantoin Ro 13-3978 is a promising drug candidate with in vivo activity superior to that of praziquantel against both adult and juvenile Schistosoma mansoni organisms. Given the drug’s contrasting low activity in vitro and the timing of its onset of action in vivo, it was postulated that immune-assisted parasite clearance could contribute to the drug’s in vivo activity. We undertook histopathological studies to investigate this hypothesis. Infected mice were treated with an effective dose of Ro 13-3978 (100 mg/kg of body weight) and were dissected before and after the drug’s in vivo onset of action. The veins and livers were excised, paraffin-embedded, and sectioned, and macrophages (IBA-1), neutrophils (Neutro), B cells (CD45R), and T cells (CD3) were stained by immunohistochemistry. For comparison, samples from infected untreated mice and mice treated with effective doses of praziquantel (400 mg/kg), oxamniquine (200 mg/kg), and mefloquine (200 mg/kg) were examined. At 24 h after treatment with Ro 13-3978, significant macrophage recruitment to the veins was observed, along with a modest increase in circulating B cells, and at 48 h, neutrophils and T cells are also present. Treatment with praziquantel and oxamniquine showed similar patterns of recruitment but with comparatively higher cellular levels, whereas mefloquine treatment resulted in minimal cell recruitment until 3 days posttreatment. Our study sheds light on the immediate immune responses to antischistosomal treatment in mice and provides further insight into immune effector mechanisms of schistosome clearance.

KEYWORDS Schistosoma mansoni, immunohistochemistry, praziquantel, mefloquine, oxamniquine, Ro 13-3978

Schistosomiasis is a chronic parasitic disease which afflicts more than 250 million people worldwide (1, 2). Behind malaria and intestinal helminthiasis, it is the third most impactful parasitic disease, where most of the disease burden is attributable to chronic inflammation and fibrosis of certain visceral organs and the resulting downstream consequences (3, 4). It is caused by trematode flatworms of the Schistosoma genus, where Schistosoma haematobium, S. japonicum, and S. mansoni are the main species affecting humans. Mature schistosomes attach to the mesenteric or urogenital vein walls and produce tens to thousands of eggs per day; the granulomas that form around these eggs are what cause the pathology (5).

The crux of the schistosomiasis control strategy is morbidity reduction using preventative chemotherapy with praziquantel, the sole drug currently available (6, 7). While the activity and safety profile of praziquantel are adequate, the drug nonetheless
carries drawbacks, such as inactivity against the juvenile stage of the worm. Importantly, the reliance on a single drug to treat hundreds of millions spurs concerns of resistance (8, 9). Treatment alternatives are therefore urgently needed.

As praziquantel was adopted as the WHO drug of choice, Hoffman-La Roche abandoned its antischistosomal lead, Ro 13-3978. A distant cousin to the obsolete antischistosomal, niridazole, Ro 13-3978 has an intriguing activity profile (10). In vivo, it has significantly superior activity to that of praziquantel against both adult and juvenile stage worms, with 50% effective dose (ED50) values of 14.6 and 138.9 mg/kg of body weight, respectively, compared to praziquantel’s ED50 of 172 to 202 mg/kg for adult worms and no appreciable effect against juvenile-stage worms. However, in vitro, it is barely active, with only moderate viability reduction after 72 h of exposure to 100 μg/ml of the drug (11–13).

Further investigations revealed this discrepancy between in vitro and in vivo activity is likely not due to drug metabolism (12). In addition, hepatic shift experiments were conducted to determine the point at which the worms get shunted to the liver as they lose viability, a rough metric for the in vivo onset of action of candidate compounds (14). Coupled with scanning electron microscope (SEM) imaging of ex vivo worms, it was shown that 24 h after drug treatment, the worms are still in the mesenteric veins of the intestines and only minor damage to the worm is visible. However, 48 h posttreatment, the worms have been shunted to the hepatic portal vein of the liver and there is extensive damage to the tegument (12). Since the liver is where adult worms mature and the temporary shunting of worms from intestinal veins to the hepatic portal veins of the liver (due to, for example, subcurative drug doses) is not itself lethal to adult schistosomes, we can rule out that the extensive damage is due to a hostile environment in the liver (14, 15).

A plausible hypothesis is that clearance could be immune assisted, as has been observed for many antischistosomal drugs, including praziquantel and oxamniquine (16). In S. mansoni-infected B-cell deficient mice, treatment with praziquantel is much less effective (17). Moreover, praziquantel and oxamniquine efficacies were shown to be markedly increased with coadministration of serum from rabbits immunized with worm membrane extract (the administration of serum alone is not effective) (18, 19). In addition, the clearance of schistosomes after treatment with praziquantel, oxamniquine, hycanthone, and some antimonials was markedly impaired in T cell-deficient (nude) mice (17, 20). However, worm clearance with chemotherapy does not always require immune assistance; in the same mouse model, mefloquine, niridazole, or amoscanate efficacy was not affected (20, 21).

In the case of Ro 13-3978, activity is also not compromised in athymic nude mice (12). However, T cell-independent humoral or innate immune components might still be triggered (22). Though a few studies have profiled medium and long-term changes to the host’s immune system after treatment of schistosomiasis with praziquantel, there are no studies that demonstrate what immune cell components are recruited at the time of worm clearance (23–25).

The aim of this study was to use immunohistochemistry to look at in situ immune responses to treatment with Ro 13-3978 and the comparator drugs. This was done by examining mesenteric vein and liver histological samples of infected and treated mice before the drugs’ onsets of action (before hepatic shift, when the worms are still in the mesenteric veins) and after the drugs’ onsets of action (after hepatic shift, when the worms have been shunted to the liver).
and comparing the histology to that from the vein and liver sections of untreated mice (infected and not infected). The design, as well as drug-specific effective doses and onsets of action, is presented in Fig. 1. All vein and liver sections were examined for each group at each dissection time point. However, changes in immune cell populations of treated mice compared to those from control mice were observable only in tissues where the schistosomes were present, i.e., in veins only at dissection 1 and in the liver only at dissection 2 (with the exception of two samples where a single worm had already been shunted to the liver at dissection 1 [examined in Discussion]). Therefore, below, we present the findings first for untreated mice and then the relevant tissue sections for each time point for the treated mice.

**Untreated mice.** In mesenteric vein sections of untreated infected mice, no particular immune responses were visibly triggered by *S. mansoni* (Fig. 2). A few macrophages were present in the adipose tissue of the mesenteries (Fig. 2A). Circulating B cells (Fig. 2C) but no circulating neutrophils or T cells were detectable (Fig. 2B and D). In uninfected untreated mice, the immune cell populations were similar to those of infected mice in the veins, with the presence of some resident tissue macrophages and B cells circulating in the blood (see Fig. S1 in the supplemental material).

In the liver, eggs triggered eosinophilia and the recruitment of B cells, T cells, and especially, macrophages, both to the granuloma and to peripheries of the hepatic veins, by which the eggs get shunted to the liver (Fig. 3). Neutrophils (Fig. 3C) were generally absent except in small clusters around granulomas (not shown). However, in contrast to those of infected mice, the liver sections of uninfected mice showed only minimal evenly dispersed populations of B cells, T cells, and macrophages (Kupffer cells) and only a trace amount of neutrophils (see Fig. S2). No accumulations of cells could be observed.

**Effect of Ro 13-3978 treatment.** Twenty-four hours after treatment with 100 mg/kg Ro 13-3978, there was significant recruitment of macrophages to the tissue areas surrounding the worms in the mesenteric veins, though macrophage attachment to the worm tegument was not observed (Fig. 4B). There was no increase in circulating neutrophils (Fig. 4C); however, the few that were present appeared to attach to the worms’ teguments (not shown), which was not seen in untreated mice. In addition,
some infiltration and attachment of B cells was observed (Fig. 4D), whereas T cells were not appreciably increased (Fig. 4E).

At 48 h posttreatment, all worms had been shunted to the liver. Inside the liver, the immune reaction appeared to follow a pattern similar to those seen in livers of untreated mice, though the numbers for each cell type were increased. The worms had already been surrounded by a dense cluster of immune cells, namely, eosinophils (Fig. 5A), macrophages, (Fig. 5B), neutrophils (Fig. 5C), B cells (Fig. 5D), and T cells (Fig. 5E). Moreover, the attachment of B cells to the worm itself was visible (Fig. 5D).
Comparison to treatment with praziquantel, oxamniquine, or mefloquine. Immune responses to treatment differed between the comparator drugs in both the mesenteric veins (Fig. 6) and the liver (Fig. 7). In the veins, at 0.5 h posttreatment with 400 mg/kg praziquantel, significant macrophage recruitment to the site of the worm was visible (Fig. 6A2). Despite the strong macrophage presence at the vein walls, very few penetrated the vein wall, and no attachment to the worm was detected. This was also observable with a treatment of 200 mg/kg oxamniquine (24 h posttreatment) (Fig. 6B2). For oxamniquine, no circulating or infiltrating neutrophils were noted;
however, some B cells and some T cells were visible that infiltrated through the mesenteric adipose tissue (Fig. 6B4 to B5). Treatment with 200 mg/kg mefloquine did not elicit strong immune responses to the worm, except for a modest recruitment of macrophages and B cells (Fig. 6C2 and 6C4, respectively). Staining for neutrophils or T cells showed no infiltration of these cells to the site of infection (Fig. 6C3 and 6C5, respectively).

At the second dissection point, in the liver, multiple immune cell populations were responsive, though at various degrees (Fig. 7). At 24 h after treatment with praziqu-
Many macrophages had been recruited to the site of the worm, and the numbers of macrophages in the liver were highly elevated (Fig. 7A2). Neutrophils were not numerous, but some attachment to the worm tegument was visible (Fig. 7A3). B cell and T cell recruitment to the vessel containing the worm were apparent (Fig. 7A4 and A5) as was some attachment of the cells to the worm (not shown).

At the time point of hepatic shift for oxamniquine treatment, 6 d after treatment, macrophages were highly present and were distributed throughout the liver tissue and

**Fig 5** Worms are shifted to the liver 48 h after treatment with Ro 13-3978, where phagocyte and leukocyte levels were elevated. (A) HE staining revealed a dense cluster of immune cells surrounding the worm, including eosinophils. (B) Most macrophages were not attached to the worm, but macrophage levels in the liver section were higher than for the untreated controls (see Fig. S2A in the supplemental material). (C) Slightly elevated levels of neutrophils in the liver tissue were notable, but there was not a large amount surrounding the worm. (D) B cell recruitment to the vessel and attachment to the worm tegument were visible. (E) T cell levels were generally slightly elevated but mostly present in egg granulomas. Abbreviations: EG, egg granuloma; EO, eosinophils; \( \delta W \), male worm.
the fibrotic tissue surrounding the worm (Fig. 7B2). B and T cell levels were moderately elevated compared to those from infected untreated control mice (Fig. 7B4 and B5). Neutrophils did not appear to be highly present around intact worms; however, worms that were disintegrating were predominantly surrounded with this cell type (Fig. 7B3; see also Fig. S3).

At 3 d posttreatment, all worms treated with 200 mg/kg mefloquine were shunted to the liver. The liver sections demonstrate only modestly elevated numbers of macrophages, neutrophils, B cells, and T cells close to vein vessels containing a shunted worm (Fig. 7C). However, as with the oxamniquine-treated mice, the samples also contained disintegrating worms with larger entourages of each cell type, especially, those with elevated neutrophilic responses (Fig. S4).

DISCUSSION

Long-term posttreatment immunomodulatory effects of antischistosomal drugs have been described, yet very little is understood about what happens as the adult worm is killed and removed, especially, at the local level (16, 24, 26, 27). The potent but slow-onset in vivo activity of Ro 13-3978, which stands in contrast to its poor in vitro activity, prompted us to investigate potential immunomodulatory effects of this drug by looking at general immune cell populations surrounding the worm using histopathology techniques. To elucidate if such effects were drug specific or a general response to worm death, we also probed the immunomodulatory activities of praziquantel, oxamniquine, and mefloquine. As these are also understudied, our work presents the first immunohistochemical examinations of the short-term local immune response to these drugs and to schistosome clearance in general.
We first examined histological vein and liver sections from infected untreated mice to serve as a reference point. In infected untreated mice, little to no immune cell recruitment to the adult worm in the mesenteric veins was observed, which is in stark contrast to the strong immune responses that the eggs elicit in the liver. This is in line with previous hematoxylin and eosin staining of histological examinations of intravascular worms (28), but is now confirmed by immunohistochemistry. Adult S. mansoni employs a variety of mechanisms to evade the hostile vascular humoral environment. These include blocking and neutralizing complement components C1q, C2, C3, C8, or C9, releasing decay-accelerating factor (DAF)-like molecules that accelerate the dissociation of C3 convertases, and producing large quantities of antioxidant proteins (superoxide dismutases, glutathione peroxidases, and peroxiredoxins) that protect against oxidative attack from monocytes and leukocytes (29–31). These complex immune evasion mechanisms are likely one reason why it is difficult to acquire immunity against this pathogen (32).

Generally, it was shown that similar cellular responses were induced regardless of treatment but to greatly variable degrees. Comparative levels of each cell type for each dissection time point (and sample type) are summarized in Table 1.

In the veins (dissection 1), treatment with Ro 13-3978 predominantly induced a significant recruitment of macrophages to the site of infection. However, this was also observed after treatment with praziquantel, oxamniquine, or mefloquine at the first dissection time point, before the hepatic shift (at 0.5 h, 24 h, and 16 h posttreatment, respectively). Nonetheless, the quantities differed greatly, with praziquantel- and oxamniquine-treated mice displaying the most acute macrophage response, whereas
Ro 13-3978-treated mice showed a marked but moderate response and mefloquine-treated mice showed only a minor response.

The macrophage recruitment levels for praziquantel before hepatic shift are particularly striking, considering that the samples are from 0.5 h posttreatment with the drug. Praziquantel has been shown to act immediately \textit{in vitro} and in a way that strongly damages the tegument and thus facilitates the attachment of antibodies to the worm tegument as soon as 1 h after treatment (18, 33). Therefore, it might be tempting to conclude that macrophage recruitment is simply proportional to the level of tegumental damage and therefore worm antigen release. Yet by this logic, mefloquine should have provoked a far stronger immune response than was observed in this study (Fig. 5). Mefloquine also acts within an hour \textit{in vitro}, extensively damaging the tegument (34). Moreover, the \textit{ex vivo} SEM studies of adult worms 24 h after treatment with 100 mg/kg Ro 13-3978 show there is a very meek morphological effect, especially, compared to those of \textit{ex vivo} worms taken 24 h after treatment with 200 mg/kg mefloquine (12, 35). Yet, treatment with Ro 13-3978 elicited stronger macrophage recruitment than treatment with mefloquine. In addition, the action of oxamniquine treatment is usually slow, requiring a few days to act on the tegument, as observed from \textit{ex vivo} worms (36). Nonetheless, macrophage recruitment to the worm was higher in mice treated with oxamniquine (Fig. 5) than in those treated with Ro 13-3978 and much higher than in those treated with mefloquine. Thus, other factors may be at play. The drugs could vary in how quickly they affect the above-described immune-evasion mechanisms or they may expose different antigens, which may be differently immunogenic, as is often demonstrated in the area of schistosome vaccinology (37). Finally, it is possible that the drugs themselves possess variable phagocyte stimulatory effects.

Responses from neutrophils, B cells, and T cells in the veins highly varied between treatments. B cell recruitment was notable for Ro 13-397-8 and oxamniquine-treated mice but less so for those treated with praziquantel and mefloquine. In the case of praziquantel, the dissection time point is likely too soon for B cell activation to be observable. Surprisingly, neutrophil recruitment to the worm was rarely observable, even though neutrophils are usually the “first responders” to an infection site. It is difficult to discern if this is due to \textit{S. mansonii}-specific factors or if it is related to the infection model, as neutrophils are known to respond differently in mice than in humans (38). Meanwhile, T cells were rarely present in the veins before the hepatic shift (except in one vein sample from a mouse treated with mefloquine) and rather were recruited to the liver after the hepatic shift.

| TABLE 1 Summary of immune cell proliferation and recruitment to the sites of infection after treatment |
|---------------------------------|-------|--------|--------|--------|
| Time point\(^a\) | Treatment | Macrophages | Neutrophils | B cells | T cells |
| Dissection 1 | | | | |
| 24 h | Ro 13-3978 | +++ | - | + | - |
| 0.5 h | Praziquantel | ++++ | - | + | - |
| 24 h | Oxamniquine | ++++ | - | ++ | tr |
| 16 h | Mefloquine | + | - | + | - |
| Infected Untreated | | tr | - | + | - |
| Dissection 2 | | | | |
| 48 h | Ro 13-3978 | +++ | + | +++ | +++ |
| 24 h | Praziquantel | ++++ | + | +++ | +++ |
| 6 days | Oxamniquine | ++++ | + | ++++ | +++ |
| 3 days | Mefloquine | + | + | ++ | + |
| Infected Untreated | | + | tr | ++ | + |

\(^a\)Posttreatment time points are for dissection 1, before the hepatic shift, when all worms are still in the veins, and for dissection 2, after the hepatic shift, when all worms have been shunted to the liver.\n
\(^b\)Valuations refer to cell types surrounding the intact worms (not eggs or decayed worms). –, no cells present; tr, a few trace cells present in some slides; +, some cells recruited; ++, moderate cell recruitment; ++++, significant cell recruitment; +++, strong cell recruitment; ++++, very strong cell recruitment.
Immune cell responses after the hepatic shift (in the liver [dissection 2]), are more difficult to compare, as egg granulomas already elicit the recruitment of all cell types to the liver even in untreated infected mice. Nonetheless, it is apparent that while treatment using any of the drugs results in elevated immune cell populations in the liver compared to that in untreated infected mice, praziquantel and oxamniquine prompted the highest recruitment of macrophages. The posthepatic shift time point for oxamniquine, 6 days, is much longer than that for the other drugs, and the large immune cell amounts could rather be an artifact of the time point. Indeed, one of the four liver samples at 24 h posttreatment of oxamniquine (dissection 1) happened to contain a single worm that had already been shunted to the liver; the immune cell recruitment in this sample was more moderate (Fig. S5 in the supplemental material), resembling that in the liver sections from Ro 13-3978-treated mice. However, the liver samples from praziquantel-treated mice were taken at a time point that was earlier than for other treatments (24 h posttreatment compared to 48 and 72 h posttreatment for Ro 13-3978 and mefloquine, respectively), and nonetheless showed much more elevated macrophage levels. Moreover, one of the four dissection 1 time point (0.5 h posttreatment) liver samples from praziquantel-treated mice already contained a shunted worm, around which a strong macrophage presence was also already evident (Fig. S6). This macrophage response after praziquantel treatment, evident in both the vein and liver samples, may be indicative of the direct immunostimulatory effects of praziquantel on macrophages and warrants further investigation.

In the liver samples from mice treated with oxamniquine and mefloquine, it was possible to observe immune responses to disintegrating and intact worms in the same sections (Fig. S3 and S4, respectively). This might be because of the slower onset of action of these drugs, where the shunting of schistosomes to the liver occurs gradually over this period. The disintegrating schistosomes were thus likely already shifted to the liver earlier on, whereas shift of the intact ones might have occurred closer to the dissection 2 time point. There was a distinctly different neutrophil response to a decaying schistosome compared to the response to intact worms (already described above), regardless of treatment, with a dense neutrophil cluster surrounding the decaying parasite. Whether the function of these neutrophils is to clear the extracellular parasite (39), facilitate wound healing (40), or stimulate or dampen adaptive immune responses (41, 42) is not known and may be worth investigating further to elucidate the general mechanisms of parasite clearance and possibly eventual acquired immunity.

In conclusion, we have described and presented the general localized host responses to treatment of a chronic S. mansoni infection in the rodent model with a panel of drugs. Taken together, our results suggest that though each drug treatment elicits an immune response, variables beyond the onset of action and quantity of antigens exposed are associated with the type and strength of the immune response after drug treatment. The treatment of S. mansoni-infected mice with Ro 13-3978 does lead to phagocytic immune responses, but the effect is milder than what is observed for praziquantel and oxamniquine. Hence, the activity of Ro 13-3978 is likely not due (at least solely) to immune-assisted clearance. Nonetheless, to characterize more precisely the time sequence in which responses are mounted and to compare drug treatments more directly, further in vivo studies using quantitative techniques, such as fluorescence-activated cell sorting (FACS), and those using in vitro cell activation cultures are required.

MATERIALS AND METHODS

S. mansoni mouse model. Female outbred NMRI mice were obtained and infected with S. mansoni cercariae as previously described (43). Briefly, mice (weight, ~20 to 22 g) were purchased from Charles River, Germany, and were allowed to acclimatize to in-house conditions (temperature, ~25°C; humidity, ~70%; 12-hour light and 12-hour dark cycle). Cercariae of S. mansoni were obtained from infected Biomphalaria glabrata snails by placing them under light for a few hours and collecting the cercarial suspension. Mice were infected subcutaneously with ~100 cercariae and allowed 7 weeks to develop an adult-stage infection.
Treatment and dissection. Seven weeks postinfection, mice \((n = 8)\) were allocated to 4 treatment arms receiving the effective doses of either Ro 13-3978 (100 mg/kg; kindly prepared by Jonathan Vennerstrom), praziquantel (400 mg/kg; Sigma-Aldrich), oxamniquine (200 mg/kg; donated by Quentin Bickle), or mefloquine (200 mg/kg; Sigma-Aldrich). All drugs were dissolved in a 70:30 Tween-ethanol (EtOH) mixture dissolved in distilled water (dH2O [10%]) right before use and were administered orally by gavage. The treatment arms were further subdivided into two dissection time points, namely, dissection 1, closely before the hepatic shift, when the worms are still in the veins, and dissection 2, after all worms are shunted to the liver. The onset of hepatic shift was previously determined and is specific for each drug (denoted and cited in Fig. 1). In addition, 4 mice were infected but not treated and 4 mice were not infected and not treated to serve as controls.

Immediately after euthanizing the mice, the livers and intestinal apparatuses from all groups were extracted. Using a dissecting microscope, sections of the mesenteric veins with or without worms inside were excised.

Histopathological analysis. The livers and vein sections were immediately placed in 50- or 15-ml BD Falcon tubes, respectively, filled with 10% neutral buffered formalin solution (4% formaldehyde; Sigma, Switzerland) for 24 h, after which they were stored at 4°C in 70% EtOH until use. Samples were embedded in paraffin, cut into 5-μm-thin sections with an HM 335 E rotary microtome (Microm International GmbH), retrieved on Superfrost Plus slides (Thermo Scientific, Germany), and allowed to dry overnight. All samples were first cut and stained with hematoxylin (Sigma) and eosin (Biosystems, Switzerland) until at least one worm per sample \((n = 4\) samples) was located.

For the staining, slides were deparaffinized and rehydrated by successive immersions in UltraClear (Biosystems), 100%, 95%, 90%, and 70% EtOH, and ddH2O. Immunohistochemical staining was undertaken to identify general immune cell populations. The primary antibodies used and their specifics are summarized in Table 2.

The protocols differed slightly depending on the antibody used. Slides were pretreated (except those for neutrophil staining) (Table 2) and internal peroxidases were blocked with 10% neutral buffered formalin solution (4% formaldehyde; Sigma, Switzerland) for 24 h, after which they were stored at 4°C in 70% EtOH until use. Samples were embedded in paraffin, cut into 5-μm-thin sections with an HM 335 E rotary microtome (Microm International GmbH), retrieved on Superfrost Plus slides (Thermo Scientific, Germany), and allowed to dry overnight. All samples were first cut and stained with hematoxylin (Sigma) and eosin (Biosystems, Switzerland) until at least one worm per sample \((n = 4\) samples) was located.

For the staining, slides were deparaffinized and rehydrated by successive immersions in UltraClear (Biosystems), 100%, 95%, 90%, and 70% EtOH, and ddH2O. Immunohistochemical staining was undertaken to identify general immune cell populations. The primary antibodies used and their specifics are summarized in Table 2.

The protocols differed slightly depending on the antibody used. Slides were pretreated (except those for neutrophil staining) (Table 2) and internal peroxidases were blocked with 3% H2O2 in phosphate-buffered saline (PBS) for 20 min. IBA-1 slides were additionally blocked with 1.5% goat serum in PBS thereafter. Slides were then incubated for 1 h with the primary antibody diluted in antibody diluent (Dako), except for the IBA-1 slides, where the antibody was diluted in PBS with 0.1% Tween. Afterwards, Histofine (Biosystems), or goat anti-rabbit IgG for the IBA-1 slides, was applied for 30 min. IBA-1 slides were additionally incubated with an avidin-biotin-peroxidase conjugate (ABC kit) for 30 min. The final development for all slides was done using the Vector NovaRed (Vector Laboratories) kit according to the manufacturer’s protocol. Meyer’s hematoxylin (Sigma) was used as a counterstain. The slides were then left to dry, mounted (Eukitt mounting medium; Sigma, Switzerland), and coverslipped (Menzel-Gläser coverslips; Thermo Fisher), and images were taken using a Leica DM5000B microscope.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/AAC.01142-17](https://doi.org/10.1128/AAC.01142-17).

**SUPPLEMENTAL FILE 1**, PDF file, 5.2 MB.

**ACKNOWLEDGMENTS**

We thank Peter Schmid, Melanie Ceci, Ernesta Damassa, Patrizia Barzaghi-Rinaudo, Vincent Romanet, and Caroline Stork from Novartis for technical support. We also thank Paul Davis and Jonathan Vennerstrom from the University of Nebraska, Omaha, for their excellent expert opinions and J. Vennerstrom for providing Ro 13-3978.

This work was supported by grants from the European Research Council (ERC...
REFERENCES


