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Mechanism for the Isotype Dependence of Antibody-Mediated Toxicity in Cryptococcus neoformans-Infected Mice

Nikoletta Lendvai,* Xiao-Wu Qu, † Wei Hsueh, † and Arturo Casadevall²*

Ab-based therapies have undergone a renaissance in recent years, but infusion-related reactions are a significant clinical problem. Administration of certain mAbs to Swiss Webster mice infected with Cryptococcus neoformans can result in acute lethal toxicity (ALT) characterized by cardiovascular collapse. The ability of a mAb to produce ALT is isotype dependent and occurs with IgG1 but not IgG3. To investigate this phenomenon, we measured spleen and liver cytokine responses and platelet-activating factor (PAF) content in mice given C. neoformans glucuronoxylomannan (GXM) followed by specific Ab of IgG1 or IgG3 isotype. We found no evidence to suggest that the differences in IgG1 and IgG3 toxicity were due to differences in chemokine or cytokine response. In contrast, liver and spleen tissue PAF content was significantly greater in mice IgG1. Furthermore, our results show differences in the response to IgG1- and IgG3-GXM complexes regarding: 1) macrophage-inflammatory protein-1α regulation, 2) splenic and hepatic PAF content, and 3) hepatic PAF content in infected mice. IgG1-associated ALT appears to be the result of greater production of PAF in response to IgG1-GXM complex formation. The results are consistent with the view that IgG1 and IgG3 interact with different Fc receptors. Our findings strongly suggest that the mechanism for Ab-mediated ALT is different from the cytokine release syndrome described after administration of other therapeutic mAbs. The Journal of Immunology, 2000, 164: 4367–4374.

Antibody-based therapies have made a major comeback, and at least a half-dozen mAbs are now licensed for clinical use against organ rejection, viral infections, inflammatory conditions, and conditions related to platelet aggregation (reviewed in Ref. 1). A persistent problem in the development and use of Ab-mediated therapies has been infusion-related reactions that range from minor to life threatening (1–3).

One infection for which Ab therapy is in advanced preclinical development is cryptococcosis. Cryptococcus neoformans is an encapsulated fungus that causes meningitis in 6–8% of AIDS patients (4). C. neoformans has a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM) (5). GXM is shed from fungal cells during infection, accumulates in body tissues (6), and may exert deleterious effects on the immune system (reviewed in Ref. 7). Passive administration of GXM-binding mAb to mice with serum GXM promotes clearance of Ag from the circulation by formation of Ag-Ab complexes that are deposited in liver and spleen reticuloendothelial cells (8–10).

Acute lethal toxicity (ALT) characterized by an acute onset of listlessness, ataxia, uncontrolled movements, and paralysis of hind limbs was described in mice with C. neoformans infection after administration of mAb to GXM (8, 11). Mice either died within an hour or made a full recovery. ALT results from cardiovascular collapse caused by hypotension and a rapid rise in hematocrit (8). According to the current model, ALT occurs when passively administered mAb binds GXM, forms Ag-Ab complexes, and triggers a capillary leak syndrome that results in hemocoagulation, hyperviscosity, and hypotension (8, 11). The trigger event is thought to be the cross-linking of Fc receptors on phagocytic cells during uptake of GXM-Ab complexes (8, 11). Fc receptor activation results in the release of platelet-activating factor (PAF) (12–15) has been implicated in the pathogenesis of ALT because PAF administration causes a similar syndrome (16–18) and ALT can be prevented by PAF antagonists (9, 11). Furthermore, PAF has been shown to be a major mediator of host damage in other models of immune complex induced disease (19–21).

ALT is an isotype-dependent phenomenon (9, 11). mAbs of IgG1, IgG2a, and IgG2b isotypes cause ALT but IgG3, IgM, and IgA do not, despite the fact that all isotypes clear GXM from serum and promote its deposition in the spleen and the liver (8, 9). The mechanism for the isotype-related differences in ALT is not understood. IgG1 mAbs presumably exert their effects in vivo primarily through cross-linking of FcyRIII receptors (22, 23). IgG3 immune complexes have been reported to bind to the Fcγm receptor, the high affinity receptor for IgG2a Abs (24). Fc receptor cross-linking can stimulate release of TNF-α and IL-1β from monocytes (25). Cross-linking of FcγII receptors on PBMCs, but not of Fcγ or FcγIII receptors, stimulates IL-1β and monocyte chemotactic protein-1 (MCP-1) release (26, 27). Immune complex-induced acute lung inflammatory injury was associated with induction of IL-1, TNF-α, macrophage-inflammatory protein-α (MIP-1α), and MIP-2 (28–31). Each of these cytokines could conceivably contribute to immune complex injury either by promoting neutrophil influx (TNF-α, IL-1, MIP-2) (28–30, 32), neutrophil activation (MIP-2) (30), inducing TNF production (MIP-1α) (31),

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or inducing PAF (TNF-α, IL-1) (28, 33). Given that both cytokines and PAF are released by Fc receptor cross-linking, we investigated their relative contribution to ALT in the context of IgG1- and IgG3-related differences in toxicity in this system.

Materials and Methods

Mice

Four- to 5-wk-old female outbred Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were used for all experiments. Outbred Swiss Webster mice from Charles River Laboratories were used in our studies because these mice have been reported to be the most susceptible to ALT (11). Mice used for chemokine studies were kept in a specific pathogen-free barrier facility in microisolator cages, fed irradiated chow, provided autoclaved bedding, and routinely monitored for serologic evidence of exposure to common murine pathogens. All serological testing was negative.

Monoclonal Abs

mAbs 3E5 IgG1 and 3E5 IgG2a are isotype switch variants of 3E5 IgG3 (34). Ascites containing mAb was obtained by paracentesis from pristane-primed mice injected with hybridoma cells. mAb was purified from ascites fluid by protein G or protein A depending on the isotype.

C. neoformans strain and GXM

Mice were infected with C. neoformans strain 24067, a virulent encapsulated strain of serotype D (American Tissue Type Collection, Manassas, VA). Strain 24067 has been used in multiple studies by several laboratories (35) and represents a serotype common in Europe (36). Strain 24067 GXM was purified from culture supernatant as described (37).

Endotoxin precautions

To minimize the possibility of endotoxin contamination, we undertook extraordinary precautions throughout all aspects of the studies described here. All work involving purification of mAb reagents or handling of reagent solutions was done by one person in a laminar flow hood. Solutions were made with endotoxin-free water or PBS. Extensive use was made of disposable pyrogen-free plasticware, pipettes, pipet tips, microcentrifuge tubes, etc. Endotoxin concentration in mAb solutions measured by Limulus Amoebocyte Assay (BioWhittaker, Walkersville, MD) was below the limit of detection of the assay. For GXM solutions, measurement of endotoxin by the Limulus assay is not practical because fungal β-glucans can produce a false positive amoebocyte lysis reaction (38). To evaluate GXM for endotoxin, we tested its ability to elicit nitrite release from IFN-γ-stimulated J774 cells as described (39). Based on the macrophage nitrite production assay, we estimate that the upper limit of endotoxin was <8 ng per 50 μg of GXM (dose used in this study). However, this reactivity could be due to either endotoxin or to another priming activity of the fungal polysaccharide on IFN-γ-stimulated J774 cells. To investigate these possibilities we passaged GXM over a polyminixin B column (Dectoxi column; Pierce, Rockford, IL) and found no difference on its ability to trigger nitrite production in the macrophage assay. We interpret this as indicating that the reactivity measured by the macrophage nitrite production assay was not due to endotoxin contamination. Samples were tested for endotoxin before and after experiments and no contamination was found.

Cytokine studies

Mice were injected i.v. with 50 μg of GXM in 0.1 ml of PBS. One hour later mice were given 300 μg of mAb i.p., either 3E5 IgG1 or 3E5 IgG3. The dose of mAb used was sufficient to clear 50 μg of GXM from the circulation and to induce ALT (8, 9). Control groups were given GXM followed by PBS, PBS followed by mAb, or PBS only. mAb was administered i.p. to avoid the possibility of intravascular precipitation of Ag-Ab complexes. Intrapерitoneal administration results in rapid diffusion to the spleen (8, 9). Mice were sacrificed 40 min after mAb administration, and spleen and liver RNA was prepared for ribonuclease protection assay (RPA). Mice were treated the same way to obtain organs for cytokine protein determination by ELISA. Groups of five mice were given GXM and mAb of either isotype or GXM followed by PBS. Control mice were given PBS and mAb or PBS only. Forty minutes after mAb administration spleens and liver were removed and homogenized in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, IN). This is because spleens were exsanguinated by the onset of anesthesia. The homogenate was centrifuged at 1500 × g for 10 min (spleen) or 20 min (liver) at 4°C to remove cell debris and the supernatant was frozen at −80°C until tested.

RPA

RNA was prepared from spleen and liver by homogenizing organs in Trizol reagent (Life Technologies, Baltimore, MD), 2 ml for spleen and 6 ml for liver, following the manufacturers’ instructions. [32P]UTP-labeled (Amersham, Arlington Heights, IL) antisense RNA transcripts were generated using mck-2b, mck-3b, mck-4, and mck-5 probe set (PharMingen, San Diego, CA) and T7 RNA polymerase (Maxima; Promega, Madison, WI). Samples were heated at 95°C for 5 min to break secondary structure. These probe sets contained probes for the detection of the following cytokines: IL-12 p35, IL-12 p40, IL-10, IL-1α, IL-1β, IL-1α, IL-1β, IFN-γ, IL-6, IFN-γ, MIF (mck-2b); TNF-α, LTβ, TNF-α, IL-6, IFN-γ, IFN-γ, TGF-β1, TGF-β2, TGF-β3, MIF (mck-3b); IL-3, IL-11, IL-7, GM-CSF, M-CSF, G-CSF, LIF, IL-6, SCF (mck-4); and Ltn, RANTES, Eotaxin, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3 (mck-5); L32 was included in all probe sets. The RPAII kit (Ambion) was used for the RPA. Approximately 20 μg of RNA from each sample was hybridized with the labeled probe at 42°C for 1 h, quenched with acetic acid, and the hybridized probe was purified. The RNA was then digested with RNase A/T1. Protected RNA was separated by denaturing 5% acrylamide/8 M urea gel (2 h at 290 V), and was visualized by autoradiography. The intensity of the bands was measured by phosphoimaging on a Storm 860 scanner and using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA) for quantitation. Samples were normalized by calculating the ratio of the signal of the band of interest (area × intensity) to that of the band corresponding to the housekeeping gene L-32 (large ribosomal subunit protein 32). Results are expressed in normalized units (n.u.).

Infection and toxicity study

C. neoformans cultures were grown at 30°C in Sabouraud dextrose broth for 2 days. Cells were washed three times in endotoxin-free PBS (BioWhittaker) and counted by Trypan blue exclusion and counted on a hemocytometer, and 5 × 103 cells were injected via the tail-vein. The inoculum was confirmed by CFU. Six days after infection mice were bled from the retroorbital sinus, and serum GXM was determined by capture ELISA as described (40), except that mAb 2H1 was used to capture GXM and mAb 2D10 was used for detection. Eight days after infection mice were injected with 0.5 mg of mAb i.p. Mice were sacrificed 40–50 min after mAb administration; spleen and liver were removed and frozen immediately in liquid nitrogen.

Measurement of PAF in organs

Spleens and livers from mice given GXM with or without Ab or Ab alone were removed, frozen immediately in liquid nitrogen, and stored at −80°C until PAF measurements were made. PAF was measured in the spleen and liver as previously described (41). Briefly, total tissue lipids were extracted by homogenizing the tissue in a 2:1 mixture of chloroform:methanol on ice. [3H]PAF (~1 KBq; Amersham) was added to the homogenate for calculation of percent recovery. After phase separation through the addition of water (20%) and centrifugation, the organic phase containing PAF was collected and dried under nitrogen flow. The lipid extract was reconstituted with 10% acetic acid with the help of 20% Tween 20 and ethanol (a final concentration of 0.15% and 1.5%, respectively). PAF was partially purified using a C18 column (Varian, Palo Alto, CA), activated with 6 ml of methanol and dried with nitrogen. After loading samples, the column was eluted with 3 ml 10% acetic acid (two times), 3 ml ethyl acetate (six times), and 2 ml of methanol (three times). The collected methanol fraction (containing PAF) was mixed with chloroform and water to a chloroform:methanol:water ratio of 1:2:0.8 and with 0.1 g of DEAE cellulose powder (Sigma, St. Louis, MO) to remove negatively charged impurities. After adjusting the chloroform:methanol:water ratio to 1:1:0.9 through further addition of water and chloroform, followed by centrifugation, the bottom phase was collected and dried. Lipid residue was dissolved in ethanol (50 μl) and reconstituted in saline containing 5 mg/ml BSA. PAF was quantified by measuring [3H]serotonin release as previously described (42). Briefly, [3H]serotonin-labeled, heparinized (100 IU/ml), and diluted (1:4 in saline) rabbit platelet-rich plasma (prepared from rabbit blood as previously described (41)) was mixed with the samples at a ratio of 500:100 μl. After 2.5 min the reaction was stopped by addition of 20 μl of 36% formaldehyde. The amount of serotonin released (calculated using the radioactivity of the supernatants and that of the whole reaction system) was used to determine the PAF content of samples based on a standard curve. PAF content of each sample was obtained by subtracting the reading of a sample duplicate containing WEB 2170, a PAF antagonist, at 20 μg/ml from that obtained by the sample only. Samples were calibrated against weight of the tissue and the extraction efficiency of PAF.

Statistics

Kruskal-Wallis test was used to compare PAF levels and cytokine levels among different treatment groups. The p values < 0.05 were considered significant.
significant. Statistical analysis was performed using the program Stat- most32 (Datamost, Sandy, UT).

Results

This study tested the hypothesis that the differences in toxicity observed for IgG1 and IgG3 administration in mice with *C. neoformans* infection were a result of differences in cytokine and/or PAF release by formation of IgG1-GXM and IgG3-GXM complexes. Because chronic infection can produce profound changes in tissue cytokine expression, we initially investigated the effects of IgG1 and IgG3 administration to uninfected mice given GXM. This allowed precise control over the amounts of GXM in the serum that can vary greatly in infected mice (8). Later studies were conducted in infected mice. The approach was to screen the cytokine response to Ag-Ab complex formation for both isotypes using RPA and then measure tissue concentrations of certain cytokines and PAF. Levels of mRNA, protein, and PAF were measured at 40 min after Ab administration because it coincides with the onset of ALT in most of IgG1-treated mice (8).

Measurement of cytokine mRNA

**Chemokines.** Mice were given GXM followed by variable region identical mAbs of IgG1 and IgG3 isotypes, and chemokine mRNA was measured in spleen homogenate by RPA. More MIP-1α and MCP-1 mRNA was present in the spleens of mice given GXM followed by IgG1 than in mice given GXM followed by IgG3 (p = 0.025 in both cases; Fig. 1). Administration of GXM followed by IgG1 also resulted in greater amounts of MIP-2 and MCP-1 mRNA compared with mice given GXM only (p = 0.020 in both cases). In contrast, mice given GXM followed by mAb of either isotype had significantly less RANTES mRNA than mice given GXM only (p = 0.039 for IgG1 and p = 0.020 for IgG3). Similarly, mice given GXM followed by IgG3 had less eotaxin mRNA than mice given GXM only (p = 0.020). The amounts of lymphotactin mRNA did not vary significantly for the various treatment groups (data not shown). TCA-3 was not detected. These results provide evidence for differential regulation of MIP-1α and MCP-1 mRNA by IgG1-GXM and IgG3-GXM complexes.

**Other cytokines.** We detected mRNA in spleen homogenate for IL-12 p35, IL-12 p40, IL-10, IL-1α, IL-1β, IL-1ra, IGIF, IL-6, IFN-γ, MIF, TNF-β, TNF-α, IL-7, M-CSF, LIF, and SCF (data not shown). We did not detect TGF-β1, TGF-β3, IL-3, IL-11, GM-CSF, or G-CSF mRNA in any of the samples examined. Among the cytokine mRNA detected, more M-CSF mRNA was found in the spleen of mice given GXM and IgG3 than in mice given GXM and IgG1 (0.107 ± 0.009 n.u. and 0.086 ± 0.017 n.u., respectively; p = 0.047). However, neither treatment resulted in RNA levels that were significantly different from that in PBS controls. Mice given GXM followed by IgG1 had more IL-6 mRNA compared with mice given PBS only (0.011 ± 0.009 n.u. and 0.004 ± 0.005 n.u., respectively; p = 0.006) but not compared with mice given GXM and IgG3. For TNF-α, IFN-γ, MIF, IL-7, and SCF, similar amounts of mRNA were found in all treatment groups (data not shown). In addition, mRNA for a number of cytokines known to play a role in regulation of inflammation was compared between untreated mice and mice given GXM followed either by IgG1 or IgG3. Compared with untreated mice, mRNA given GXM followed by mAb had more IL-1β (0.016 ± 0.003 n.u. and 0.162 ± 0.082 n.u., respectively; p = 0.014), IL-1a (0.021 ± 0.007 n.u. and 0.053 ± 0.031 n.u., respectively; p = 0.014), and IL-1ra (0.008 ± 0.001 n.u. and 0.034 ± 0.027 n.u., respectively; p = 0.014) mRNA. In contrast, compared with untreated mice, mice given GXM followed by mAb had less IL-12 mRNA (0.016 ± 0.008 n.u. and 0.009 ± 0.002 n.u., respectively, for IL-12 p35 and 0.004 ± 0.002 n.u. and 0.001 ± 0.001 n.u., respectively, for IL-12 p40; p = 0.05 for both). Similar amounts of IGIF and IL-10 mRNA were present in the spleens of mice in both groups. We found no significant difference in the level of mRNA for any of the above cytokines in response to IgG1-GXM complex formation relative to IgG3-GXM complex formation.

**Individual variation in Swiss Webster mice cytokine expression.** The RPA analysis revealed no apparent correlation between cytokine mRNA and the treatment administered for certain cytokines in individual mice. This was particularly striking for LTβ and IP-10. Six of 26 mice examined had high levels of IP-10, including 3 of 6 mice given PBS only; the remaining 20 mice had very low amounts of IP-10. Seven of 24 mice had high levels of LTβ including 2 of 6 mice given PBS only. LTβ was minimal in the remaining 17 mice. Hence, it appeared that Swiss Webster mice might have a limited number of baseline cytokine expression patterns that are independent of the treatment received, because some mice given only PBS expressed these cytokines as well. Acute changes in the expression of other cytokines (e.g., IL-6) from the actual intervention (administration of GXM and/or mAb) occurred against this background of cytokine expression.
Measurement of cytokine protein

For selected cytokines the protein concentration in liver and spleen was measured 40 min after treatment with Ag, Ab, or both.

**MIP-1α, MIP-2, and MCP-1.** MIP-1α, MIP-2, and MCP-1 protein production was measured in the liver and spleen after administration of GXM followed by IgG1 or IgG3. As shown in Fig. 2, GXM by itself induced coordinate expression of MIP-1α, MIP-2, and MCP-1 in the liver, and MIP-2 and MCP-1 in the spleen. Administration of GXM followed by IgG3 mAb resulted in greater production of MIP-1α in liver tissue of mice relative to mice given GXM alone (p < 0.016). In all other instances there was no significant difference between the tissue concentration of these chemokines in mice given GXM alone or GXM and either mAb. Administration of GXM followed by IgG1 did not result in chemokine levels that were significantly different from those measured in mice given GXM followed by IgG3. Consequently, differences in the production of these chemokines are unlikely to be responsible for the differential ability of IgG1 and IgG3 Abs to produce ALT.

**IL-1β, IL-6, and TNF-α.** Because IL-1β, IL-6, and TNF-α has been associated with immune complex injury, we investigated whether IgG1 and IgG3 immune complexes regulated these cytokines differentially. IL-6, TNF-α, and IL-1β were detectable in the spleen of each mouse examined (Fig. 3). GXM administration did not significantly increase expression of any of these cytokines in the spleen of mice compared with control mice. In contrast, administration of GXM alone resulted in higher levels of all three cytokines in the liver of mice compared with controls. Administration of GXM followed by mAb did not result in significantly different splenic cytokine levels compared with controls given mAb only. Compared with cytokine levels in mice given GXM only, mAb administration following GXM resulted in higher hepatic IL-1β levels, when IgG3 was given (p = 0.028), and higher hepatic IL-6 levels, when mAb of either isotype was given (p = 0.016 for IgG3, p = 0.014 for IgG1). Only the hepatic IL-1β levels varied significantly depending on the isotype of mAb given following GXM administration, with IgG3 administration resulting in greater IL-1β production than IgG1 administration (p = 0.014). These results show that GXM preparations can induce the production of IL-1β, IL-6, and TNF-α in the liver. Further, because IgG1 administration following GXM was not associated with an increase in any of these cytokines, it is unlikely that they are associated with the differences in the ability of IgG1 and IgG3 to mediate ALT.
mice. To investigate this possibility we administered IgG1 or IgG3 mAb to infected mice and measured liver and spleen tissue PAF concentration. Serum GXM concentration of the infected mice on day 6 and was 1.5–58.0 μg/ml, a range associated with ALT after IgG1 administration (8). Seven of eight C. neoformans-infected mice given IgG1 showed signs of ALT and one died of after IgG1 administration. Infected mice given IgG1 had higher PAF concentration in both their spleen and liver tissue compared with infected mice given IgG3 (p = 0.013 and p = 0.008, respectively, Fig. 4, C and D). The difference in hepatic PAF concentration between infected mice given IgG1 vs IgG3 was nearly 8-fold. PAF concentration in the spleen and liver of infected mice given IgG3 was not significantly different from that in uninfected mice given IgG3 only. In infected mice given IgG1, the liver was the main source of PAF, with the mean PAF concentration being over twice that in the spleen. Furthermore, mean hepatic PAF concentration in infected mice given IgG1 was nearly three times that in uninfected mice given GXM and IgG1.

Discussion

Previous studies have shown that the mechanism of ALT 1) involves Fc receptor cross-linking, 2) can be inhibited by antagonists of PAF or ablation of macrophages, and 3) produces severe and rapid hemoconcentration and hypotension resulting in cardiovascular collapse (9, 11). The phenomenon of ALT has been observed with several IgG1 mAbs to GXM (8, 11). Fc receptor activation has profound and diverse consequences in cellular physiology, including mediating alterations in cytokine production and PAF release (14, 21, 25, 28, 29, 43). The relative contribution of these effects to ALT is unknown. To investigate the mediators of ALT we studied cytokine and PAF release in a model in which Swiss Webster mice were given GXM followed by either IgG1 or IgG3.

Administration of GXM followed by IgG1 or IgG3 had different effects on MIP-1α, MCP-1, and M-CSF mRNA levels in spleen cells. MIP-1α and MCP-1 mRNA was present in greater quantities in the spleen of mice given GXM followed by IgG1. However, no difference in MIP-1α and MCP-1 tissue protein concentration was measured by ELISA. The most likely explanation for this observation is the time of the experiment. Measurements for both mRNA and protein were made 40 min after Ab administration, a time that coincides with the onset of ALT in the majority of mice (8). This time interval may be sufficient for alteration of mRNA levels but not for detecting differences in tissue protein concentration. Cytokine protein studies at later times may reveal differences in protein that correlate to the cytokine mRNA changes measured. However, the interpretation of such measurements would be complicated by the fact that the release of PAF and the internalization of GXM complexes may have additional uncontrolled effects on cytokine expression independent of Fc receptor activation. For our purposes, the differences in IgG1 and IgG3 effects on MIP-1α, MCP-1, and M-CSF do not appear to be of consequence for the phenomenon of ALT. We also examined expression of MIP-1α, MIP-2, MCP-1, IL-1β, IL-6, and TNF-α in the spleen and liver of mice given GXM and GXM-specific mAb. Each of these cytokines is released in response to immune complexes (26, 28–31, 44). We did not find evidence that any of these cytokines were expressed in greater quantities following GXM and IgG1 administration compared with GXM and IgG3 administration.

In contrast, administration of IgG1 and IgG3 mAbs resulted in significant differences in PAF production. Administration of GXM followed by IgG1 or IgG2a produced greater tissue PAF concentration in both spleen and liver than administration of GXM followed by IgG3. To determine whether this phenomenon occurred in infected mice, PAF was measured in the spleen and liver of

Measurement of PAF in mice given GXM and specific mAb.

PAF was measured in the spleen and liver of mice given GXM followed by 3E5 IgG1, IgG2a, or IgG3 mAb to determine whether the various treatments produced differences in tissue PAF. Administration of IgG1 to mice given GXM resulted in greater concentration of PAF in both the liver and spleen than IgG3 administration (p = 0.021 for liver and spleen, Fig. 4, A and B). Similarly, mice given GXM followed by a mAb associated with ALT (either IgG1 or IgG2a) had higher PAF concentration in their spleen and liver tissue compared with mice given GXM followed by IgG3 (p = 0.042 for liver and spleen). Mice given GXM and IgG3 had higher PAF concentration in spleen tissue but not in their liver tissue relative to untreated mice. In considering these results, it is noteworthy that the average weight of the liver is ~10 times that of the spleen. Hence, although the PAF concentration in the spleen tissue of mice given GXM and IgG1 was 3 times that in their liver, most of the PAF was still made in the liver.

Measurement of PAF in infected mice given GXM-specific mAb.

Because ALT was observed in infected mice given IgG1 but not in mice injected with GXM followed by IgG1, we hypothesized that more PAF was released by IgG1-GXM complexes in infected mice. To investigate this possibility we administered IgG1 or IgG3

![FIGURE 4.](image-url)
infected mice given either IgG1 or IgG3. Infected mice given IgG1 mAb had nearly 8-fold more PAF in their spleen and liver than mice given IgG3 mAb. Thus, it is likely that differential regulation of PAF is responsible for the ALT that follows IgG1 administration but not IgG3 mAb administration. In other pathologic conditions characterized by hypotension, cardiovascular collapse has been associated with the production of NO (45). However inhibition of NO synthase does not prevent IgG1-mediated ALT (11), a finding that argues against a critical role for NO in this phenomenon.

ALT occurred in *C. neoformans*-infected mice given IgG1 but not in mice given GXM followed by IgG1. Although the basis for the differential susceptibility of infected and noninfected mice to ALT was not specifically investigated in this study there are several possible explanations for this phenomenon. In mice given GXM followed by IgG1, PAF content per gram of spleen tissue was nearly three times greater than PAF content per gm of liver tissue. However, in infected mice, PAF content per gram of liver tissue was 2.6 times greater than PAF content per gram of spleen tissue. Because the liver weighs about 10 times that of the spleen of mice this translates to the presence of twice as much PAF in infected mice than in mice given GXM and IgG1. A 2-fold difference in PAF tissue content may be biologically significant given that a linear relationship has been reported between liver PAF and vascular permeability at the PAF tissue concentrations measured in this study (20). These results suggest that one plausible explanation for the fact that ALT was observed in infected mice given IgG1 but not in uninfected mice given GXM plus IgG1 is greater PAF production in response to IgG1-GXM complexes in infected mice. In addition, mice infected with *C. neoformans* may be more responsive as a consequence of concurrent infection. The mechanisms responsible for the differences in susceptibility to ALT for infected and noninfected mice and large increase in hepatic PAF tissue concentration in infected mice in response to IgG1-GXM complexes remains to be investigated.

PAF has been measured in organs after infusion of Ig aggregates but to our knowledge, this is the first study where PAF was measured in the liver and spleen after in vivo generation of immune complexes. Our measurements of PAF in the spleen and liver of mice with ALT also distinguish the pathophysiology of ALT from that of endotoxin mediated shock that is also in large part mediated by PAF. In endotoxin mediated shock, induced in rats by administration of 10 mg kg$^{-1}$ LPS i.v., the spleen was the major source of PAF (46). No PAF production was detected in the liver in response to LPS (46). In contrast, in IgG1-mediated ALT the liver was the major source of PAF.

The mechanism of ALT in mice appears to be different from the infusion related toxicity observed with certain mAbs which has been ascribed to a cytokine release syndrome. For OKT3, a murine IgG2a mAb to CD3 used in immunosuppressive therapy to prevent organ allograft rejection, infusion related reactions have been associated with an increase in IL-2, IL-6, TNF-$\alpha$, and IFN-$\gamma$ levels (3). Similarly, CAMPATH 1-H, a humanized IgG mAb that recognizes CD52 used for the treatment of leukemia, is associated with TNF-$\alpha$, IFN-$\gamma$, and IL-6 release (2). For both of these therapeutic mAbs cytokine release is thought to be a consequence of cross-linking Fc receptors on human leukocytes (2).

Our observations are also relevant to the question of whether IgG1 and IgG3 interact with different Fc receptor. Diamond and Yelton (47) reported in 1981 a receptor on mouse macrophages that bound the Fc portion of IgG3 but not the other IgG subclasses. mAbs to *C. neoformans* GXM revealed that mAbs of the IgG1, IgG2a, and IgG2b isotype are protective against systemic infection in mice while IgG3 is not, an observation also consistent with the existence of a different receptor for IgG3 (34, 48). Yuan et al. (49) demonstrated that in $\gamma$-chain knockout mice with defective Fc$\gamma$I and Fc$\gamma$II receptors IgG1, IgG2a, and IgG2b were no longer protective, whereas IgG3 remained disease enhancing. However, Gavin et al. (24) reported the binding of IgG3 immune complexes by Fc$\gamma$RII, the high affinity receptor for IgG2a. Their results indicated that Fc$\gamma$RI that binds monomeric IgG2a with high affinity can bind IgG3 immune complexes with a lower affinity. IgG2a can cause ALT similar to that caused by IgG1 (11), but IgG3 has no toxicity (8). In this paper we show that both IgG1 and IgG2a induce higher levels of tissue PAF than IgG3. Because all subclasses of IgG can clear serum GXM (9), the most straightforward interpretation of our data is that IgG3 interacts with a different Fc receptor than IgG1 or IgG2a.

MIP-2 and MCP-1 protein were present in greater quantities in mice given GXM compared with control mice, though no significant difference between these groups was detected in terms of splenic mRNA. This suggests that GXM does not produce increased transcription of these chemokines, but does increase their expression. GXM may act posttranscriptionally to either increase translation or to increase posttranslational modification resulting in greater amounts of MIP-2 and MCP-1 tissue protein. Alternatively, GXM may in some manner prolong the half-life of these molecules in tissue. Although many investigators have demonstrated that GXM can induce cytokine production from leukocytes in vitro (50–53), in vivo cytokine production in response to purified GXM alone has not been previously reported. Our results indicate that GXM administration leads to expression of MIP-2 and MCP-1 in the spleen and liver, and MIP-1$\alpha$, IL-1$\beta$, IL-6, and TNF-$\alpha$ in the liver only. The ability of GXM to induce coordinate in vivo expression of these cytokines involved in regulation of inflammatory reactions underscores its potential role as an immunomodulator.

During these studies the possibility of endotoxin contamination was a daily concern. Endotoxin is known to have profound effects on cytokine production, and injection of endotoxin-contaminated solutions could have interfered with our studies. We are confident, however, that the effects measured here are not the result of endotoxin contamination because 1) extraordinary precautions were taken during all phases of the study to maintain LPS-free conditions, 2) the amount of LPS reported in the literature that is necessary to elicit a significant cytokine or chemokine response in mice reported is ~1000 times higher than the maximum possible LPS contamination in our solutions (54–59); and 3) the pattern of cytokine mRNA induction observed does not correlate with that reported for LPS effects (59). Instead, our results are consistent with cytokine release because of either direct GXM effects on mouse cells as described in vitro (see above) and/or stimulation of Fc receptors by Ag-Ab complexes.

We noted individual variation in baseline cytokine expression among our mice. Swiss Webster mice are outbred and this could reflect mouse-to-mouse differences in baseline cytokine expression. It should be noted that, for reasons not presently understood, only 40–50% of *C. neoformans* infected mice given GXM-specific 18B7 IgG1 mAb develop severe toxicity and die. Whether the differences in baseline cytokine expression, or possibly other differences not observed by us, contribute to ALT in some mice and not in others treated the same way should be a future avenue of investigation. Alternatively, differential susceptibility to ALT may be due to differential sensitivity to PAF, which has been reported among different strains of mice and among Swiss Webster mice from different suppliers (60).

In summary, we found 1) that GXM can induce coordinate expression of MIP-2 and MCP-1 in the spleen and liver, and MIP-1$\alpha$, IL-1$\beta$, IL-6, and TNF-$\alpha$ in the liver only; 2) that GXM-IgG1 and
GXM-IgG3 complexes differentially regulate MIP-1α and MCP-1 mRNA; 3) no evidence that differential cytokine regulation is responsible for the isotype-dependence of ALT; 4) differential regulation of PAF production by GXM-IgG1 and GXM-IgG3; and 5) significantly higher PAF levels in infected mice given IgG1 than in uninfected mice given GXM followed by IgG1. Our results strongly suggest that isotype dependence of ALT is due to differential regulation of tissue PAF content by GXM-IgG1 vs. GXM-IgG3 complexes. Furthermore, our data indicate that ALT is likely to be fundamentally different from cytokine release syndromes in being mediated by PAF and not by cytokines.

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