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Fc Receptors Are Not Required for Antibody-Mediated Protection Against Lethal Malaria Challenge in a Mouse Model

Harris L. Rotman,* Thomas M. Daly,* Raphael Clynes,† and Carole A. Long2*

The mechanisms by which Abs mediate protection during blood-stage malaria infections is controversial, with some evidence pointing to the direct effect of Abs on parasite invasion and growth, while other studies suggest that Abs act in cooperation with monocytes to achieve parasite inhibition. To determine whether the effector phase of protection in vivo to the rodent parasite Plasmodium yoelii yoelii requires Fc receptor bearing cells, we passively transferred immune sera into Fcγ-chain knockout mice. Inflammatory macrophages from these knockout mice were unable to mediate phagocytosis or Ab-dependent cell-mediated cytotoxicity (ADCC) through FcγRI, FcγRII, or FcγRIII. Passive transfer of either P. y. yoelii hyperimmune sera or anti-GST-PyC2 sera directed to the major merozoite surface protein (MSP-1) of this parasite enabled both BALB/cByJ mice and Fcγ-chain-deficient mice to resist lethal P. y. yoelii 17XL (Py17XL) challenge. mAb302, a protective IgG3 Ab, also passively protected both strains of mice. Most of these samples contain Ab isotypes that would not be able to protect mice if their protective effects required Ab-dependent cell-mediated cytotoxicity. These results establish that, in this infection, protection is directly mediated by Abs and does not require the participation of Fc receptors. The Journal of Immunology, 1998, 161: 1908 –1912.

In many of the most heavily populated areas of the world, malaria continues to be a major source of morbidity and mortality. It is estimated that 300 to 500 million clinical cases of malaria occur each year, resulting in up to 2.7 million deaths (1). Unlike several other acute diseases, immunity to malaria occurs only after many years of recurring infections. This immunity is not complete, since protection is defined as lessened disease symptoms and lower levels of parasites in the blood stream.

Humoral immunity plays an important role in host defense against the erythrocytic stages of human, simian, and rodent malarial (2–4). IgG isotypes have been implicated as important components of this acquired immunity, since passive transfer of human, monkey, or rodent IgGs can provide significant protection to naive recipients from malarial infection (2, 5–9). In addition, studies with immunodeficient mice have shown that resolution of infection with the rodent parasite Plasmodium yoelii yoelii requires humoral immunity (4). Transfer of hyperimmune serum (10) or of various mAbs, such as mAb302 (8–9), can impart passive immunity to mice against a normally lethal challenge with P. y. yoelii. Moreover, mice immunized with a fusion protein (GST-PyC2) encoding the carboxyl region of P. y. yoelii merozoite surface protein-1 (MSP-1)3 also survive parasite challenge (11–13). Previous studies from our laboratory (13, 14), which have been confirmed by others (15), established that this protection is primarily mediated by Abs.

The mechanisms whereby Abs can transfer protective immunity to malaria are not well understood. Some evidence indicates that the main protective role of Ab is to interfere with the merozoite invasion of RBC (16–18). Abs have also been found to interfere with intraerythrocytic development of the parasite in vitro studies (19). In contrast, studies using human immune sera demonstrated that passive protective activity of human immune globulins could not be correlated with inhibition of either penetration or intraerythrocytic development of Plasmodium falciparum parasites. Instead, protective activity was reported to correlate with an in vitro Ab-dependent cellular inhibitory (ADCI) assay involving mononuclear cells. These results suggest that protective IgG isotypes do not function independently but rather through interaction with FcRs found on mononuclear cells (20).

FcRs are surface glycoproteins known to be widely distributed on cells of the immune system, and, by binding IgG and IgE, to couple humoral and cellular responses (21). FcγRI, found on monocytes/macrophages and neutrophils, is the high affinity IgG receptor. By binding monomeric IgG, FcγRI mediates Ab-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis after Ab cross-linking. FcγRII and FcγRIII are low affinity IgG receptors, also found on many cells of the immune system, and are responsible for triggering ADCC, phagocytosis, and the release of inflammatory mediators when cross-linked by immune complexes. In mice, FcγRI binds IgG2a Abs preferentially, while FcγRII and FcγRIII have higher affinities for the IgGl and IgG2b isotypes. Mouse IgG3 was reported recently to bind with low affinity to FcγRI on macrophages (22) but may bind to a fourth, independent IgG3-specific receptor (23–24).

A subunit of FcγRIII and FcεRI, the γ-chain, is required for efficient cell surface expression of these receptors as well as signal

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3 Abbreviations used in this paper: MSP-1, merozoite surface protein-1; Py17XL, Plasmodium yoelii yoelii 17XL; PyRHS, Plasmodium yoelii yoelii 17XL hyperimmune serum; pRBC, parasitized RBC; GST, glutathione S-transferase of Schistosoma japonicum; RAS, Ribi adjuvant system; ADCC, Ab-dependent cell-mediated cytotoxicity.
transduction by these FcRs (21, 25). A mouse strain genetically deficient in the γ subunit lacks FcγRIII and FcεRI expression on NK cells, macrophages, and mast cells (26). Macrophages from these mice may have diminished expression of FcγRI, and IgG2a binding does not occur through this receptor, indicating a functional requirement for the γ subunit. Though not required for ligand binding or surface expression in transfected fibroblasts, the γ-chain has been found to be associated with FcγRI in a human cell line, where it may function as a signal-transducing subunit (27). No evidence to date indicates that the γ-chain associates with FcγRII, yet in FcR γ-chain-deficient mice the contribution of this receptor to macrophage phagocytic function also is lost (26). Thus, inflammatory macrophages from these knockout mice were unable to mediate phagocytosis or ADCC through FcγRI, FcγRII, or FcγRIII (26). To illuminate the effector mechanisms by which Abs mediate protection against malaria, we utilized these FcR γ-deficient mice to determine whether Abs inhibit parasitemias in mice directly or require FcRs on host mononuclear cells.

Materials and Methods

Experimental animals and parasites

Six- to eight-week-old male BALB/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and FcR γ-chain-deficient mice (BALB/c background, both +/+ and −/−) were raised at Taconic Farms (Germantown, NY) after 12 successive backcrosses to generate congenic lines. The lethal P. y. yoelii 17XL strain (Py17XL) was maintained as described previously (11, 13). Challenge infections were initiated by i.v. injection of 1 × 10^8 erythrocytes parasitized with the lethal variant, Py17XL. The course of infection was monitored by microscopic examination of stained blood films.

Recombinant construct and fusion protein

The recombinant construct and resultant fusion protein were described previously (11). Briefly, the carboxyl-terminal region of the P. y. yoelii 17XL MSP-1 gene was PCR amplified and joined in frame to the 3′ end of the Schistosoma japonicum glutathione S-transferase gene (GST) within the pGEX2T vector. The resultant fusion protein, designated GST-PyC2, was expressed in Escherichia coli and isolated by affinity chromatography. The PyC2 portion of the fusion protein was isolated from GST-PyC2 while bound to glutathione-agarose (Sigma, St. Louis, MO) with thrombin (ICN Biochemicals, Costa Mesa, CA).

Abs and immune sera

To generate polyclonal sera to PyC2 Ag, BALB/cByJ mice were immunized with GST-PyC2 recombinant fusion protein as described previously (11). Briefly, 5-mo-old BALB/c mice were immunized with 75 μg of GST-PyC2 (providing 25 μg of PyC2) administered s.c. in 200 μl (two sites, 100 μl each site) of Ribi adjuvant system (RAS, Ribi Immunocchemical Research Laboratories, Hamilton, MT) suspended in PBS. All mice were boosted s.c. with an equivalent dose at 3 wk and i.p. at 6 wk and 9 wk after the first inoculation. Eleven wk after the first inoculation, these immunized mice were exsanguinated, and serum was isolated. Anti-GST-PyC2 serum was also generated by using alum (Imject Alum; Pierce, Rockford, IL) as an adjuvant and administered in three s.c. injections by the schedule described above (using 60 μg of protein per dose, providing 20 μg of GST-PyC2). A hyperimmune serum (PyHIS) was prepared in BALB/cByJ mice by repeated infection as described previously (28). mAb 302 was used directly from ascites fluid, at a concentration of 1 mg/ml as determined by radial immunodiffusion in agarose. All sera and ascites preparations were stored at −20°C.

ELISA

ELISAs were performed as described (13, 14). Briefly, wells of Maxi-sorb immunoplates (Nunc, Naperville, IL) were coated with PyC2 at 1 μg/ml in carbonate buffer (pH 9.6). Wells were blocked with 0.2% Tween 20 (Sigma) in 25 mM Tris-HCl (pH 8.0)-150 mM NaCl (Tris-buffered saline). Dilutions were made as indicated in 0.1% Tween 20-Tris buffered saline and diluted to wells in duplicate. For isotype analysis, bound Abs were detected with affinity-purified, biotinylated rabbit anti-mouse (IgG1, IgG2a, IgG2b, IgG3, or IgM) Abs (Zymed Laboratories, South San Francisco, CA), avidin-alkaline phosphatase (Zymed), and p-nitrophenyl phosphate (Sigma 104; Sigma). Isotype-specific reagents were shown to possess minimal cross-reactivity, and their relative concentrations were adjusted to provide equivalent levels of reactivity against mAbs of different isotypes before analysis of sera and ascites fluid. Isotype distribution assays were terminated by addition of 5 M NaOH (50 μl per well) and read at 405 nm using a Bio-Tek (Winooski, VT) instruments EL308 EIA plate reader.

Challenge infection and transfer of immunity with serum and IgG

One day before parasite challenge (day −1), groups of naive BALB/cByJ (+/+), or FcR γ-chain knockout (−/−) mice were given 0.5 ml of PyHIS normal mouse serum, mAb 302 ascites fluid, or polyclonal anti-GST-PyC2 serum by i.p. injection. An equal dose was administered to all groups on days 0 and +1 relative to challenge infection. In some experiments, mAb 302 ascites fluid was diluted by 1:6 or 1:64 in PBS, as indicated. Infection control animals were untreated.

Results

Groups of naive BALB/cByJ (+/+), or FcR γ-chain-deficient (−/−) mice on a BALB/c genetic background were given 0.5 ml of either polyclonal anti-GST-PyC2 serum, mAb 302 ascites fluid, or polyclonal PyHIS by i.p. injection on days −1, 0, and +1 relative to challenge with Py17XL. As shown in Figure 1, animals receiving only the parasite challenge rapidly succumbed to infection, with all animals but one having greater than 50% parasitemia by day 8 in both wild-type mice and FcR γ-chain-deficient mice. One infection control BALB/cByJ mouse had a delayed onset of parasitemia (the animal became patent on day 6) but eventually
was removed from the study by day 13 due to high parasitemia. In contrast, mice given PyHIS did not develop patent parasitemias. Both immunocompetent and knockout mice were completely protected and showed no parasitemias up to day 18, when the experiment was terminated (Fig. 1). Those mice given anti-GST-PyC2 serum clearly demonstrated a delay in the onset of a patent parasitemia and a protracted period of parasite inhibition, at levels normally seen when using this sera (13). We have previously shown that mice immunized with GST alone in RAS at equivalent amounts to that used in GST-PyC2 immunizations were not protected from lethal malarial challenge (11), and passive transfer of anti-GST sera also did not elicit any degree of protection (13). Therefore, the protection seen can be attributed only to the anti-PyC2 specificities. When anti-GST-PyC2 sera was given to infected animals, both wild-type and knockout mice developed patent parasitemias by day 6, and there was no significant difference in the levels of parasitemia at any given time point between these two strains of mice, except on day 10. Surviving mice had no detectable parasitemias by day 18 in both groups. Thus, the effectiveness of the polyclonal anti-GST-PyC2 serum in mediating protection was equivalent in both BALB/cByJ (+/+) mice and FcR \( \gamma \)-chain-deficient (−/−) mice. Finally, mAb 302 (an IgG3 isotype) ascites fluid was also able to inhibit patent parasitemia in both mouse strains, with no parasites detected in blood films for 18 days after inoculation (Fig. 1). This last result was anticipated, since IgG3 isotypes may still bind to specific Fc receptors even in the knockout mice (23–24). To determine the effects of serum transfer in \( \gamma \)-chain heterozygous mice, these mice were also infected with Py17XL and given anti-GST-PyC2 sera, PyHIS, and mAb 302. FcR \( \gamma \)-chain-heterozygous (+/−) mice exhibited the same parasitemia curves after serum transfer as the (−/−) knockout mice (data not shown).

To assess the distribution of anti-malarial isotypes in the various sera samples, the isotype distribution of Abs specific for PyC2 was determined by ELISA for anti-GST-PyC2 polyclonal sera, PyHIS, and mAb 302 ascites fluid (Fig. 2). All samples were diluted 1:500. Normal mouse serum had no reactivity to PyC2 Ag (data not shown). mAb 302 is of the IgG3 isotype and so had no reactivity in ELISA when secondary Abs other than anti-IgG3 were used. In anti-GST-PyC2 serum, there is a predominance of IgG1, IgG2a, and IgG2b isotypes, with lesser amounts of IgG3 and IgM (14). These results contrast with the isotype distribution of PyC2-specific Abs in P. y. yoelii 17XL hyperimmune serum, which is predominantly IgG1, IgG2a, and IgG3, with lesser amounts of IgG2b and IgM, an observation in agreement with earlier assessments of isotype distribution in these sera (13, 14). In hyperimmune serum, antimalarial Abs of the IgG2a isotype have previously been found to modulate parasitemias in mice infected with P. y. yoelii.

To approach this question in another way, we prepared anti-GST-PyC2 serum by immunization of naive mice with GST-PyC2...
FIGURE 4. Passive immunity imparted by transfer of anti-GST-PyC2 sera generated by immunization in alum. Naive BALB/cByJ mice were given 0.5 ml of polyclonal anti-GST-PyC2 serum (○) or normal mouse serum (□) 1 day before parasite challenge with Py17XL. This dose of serum was repeated on the 2 subsequent days. Naive FcR γ-chain (−/−) knockout mice were given 0.5 ml of polyclonal anti-GST-PyC2 serum (▼) or normal mouse serum (△) 1 day before parasite challenge, and this dose was repeated for 2 days. n = 4 for all groups. r = removed, due to high parasitemia. Data shown are the means ± SE of the percent parasitemia on each day.

in alum, since this adjuvant elicits a different isotype profile than serum generated by using other adjuvants (14). When alum is used, the PyC2-specific Abs are almost exclusively of the IgG1 isotype, with negligible levels of IgG2a, IgG2b, IgG3, and IgM. Groups of naive BALB/cByJ mice or FcR γ-chain-deficient mice (−/−) were given 0.5 ml of this alum-generated polyclonal anti-GST-PyC2 serum or normal mouse serum by i.p. injection on days −1, 0, and +1 relative to parasite challenge with Py17XL. As shown in Figure 4, animals receiving normal mouse sera rapidly succumbed to infection, with all animals having greater than 50% parasitemia by day 6 in both BALB/cByJ mice and FcR γ-chain-deficient mice. In contrast, those mice given anti-GST-PyC2 serum generated with alum clearly demonstrated a delay in the onset of a patent parasitemia and a protracted period of parasite inhibition. Though the levels of protection seen when using alum-generated sera are quite variable in each individual mouse (Fig. 4), they are quite significant, since all mice given alum-generated sera have a delay in parasitemia levels by at least 1 whole day when compared with infection control animals. When anti-GST-PyC2 sera was given to infected animals, both wild-type and knockout mice developed patent parasitemias by day 4, and there was no significant difference in the levels of parasitemia at any given time point between these two strains of mice from days 4 to 8. Thus, the effectiveness of the alum-generated polyclonal anti-GST-PyC2 serum in mediating protection was similar in both BALB/cByJ (+/+), mice and FcR γ-chain-deficient (−/−) mice, supporting the interpretation that IgG3 isotypes are not solely responsible for generating the protection seen in these studies.

Discussion

Observations in humans (2, 5–6), monkeys (3, 7), and rodents (4) indicate that passive transfer of immune serum can prevent parasitemia from rising after challenge with infected erythrocytes or cause a fall in an existing parasitemia. The mode of action of Abs remains controversial. Possible mechanisms include inhibition of merozoite invasion of erythrocytes (16–18), inhibition of intraerythrocytic development of parasites (19), and opsonization and uptake of infected erythrocytes and/or merozoites (20). In this study, we have examined the effects of the transfer of hyperimmune serum, anti-GST-PyC2 serum, and mAb 302 ascites fluids to nonimmune mice that are genetically deficient in the γ subunit found in several FcRs on monocytes/macrophages and other cells of the immune system. These FcR γ-chain knockout mice are profoundly immunocompromised and cannot mediate phagocytosis or ADCC through FcγRI, FcγRII, or FcγRIII (26). We found no differences in the inhibition of parasitemia by various protective polyclonal and mAbs in wild-type and FcR γ-chain knockout mice, indicating that FcR-mediated phagocytosis and ADCC are not essential mechanisms in parasite clearance in this model system. The sera used in these studies showed great variability in isotype compositions, and though some sera protected against lethal challenge better than others, equivalent protection was seen in all mice given similar sera, whether they were immunocompetent mice or knockout mice.

We sought to exclude the possible protective role of IgG3 in polyclonal sera in two types of experiments. The first used alum as an adjuvant, a procedure that resulted in the production of predominantly IgG1 Abs. The second approach was to compare the amount of IgG3 Abs in the polyclonal sera with the protective activity of a similar amount of a monoclonal IgG3 (mAb 302). It is possible that polyclonal IgG3s may be of higher affinity or of superior specificity to the mAb 302 and that the small amounts of these isotypes present in polyclonal sera are responsible for all of the protection seen in passive transfers using these sera. However, many efforts have been made by ourselves and others to produce mAbs against this region of the MSP-1 molecule, and most of the Abs generated have no protective activity (data not shown). Thus, superior Abs have not been found by cell fusions. We believe that this comparison is a valid experimental system since, in view of the above, it is highly unlikely that the polyclonal Abs to PyC2 (many of which have no biologic activity at all) are superior to mAb 302 in passive protection.

There may be differences in the mechanisms of Ab-mediated parasite clearance in humans as compared with murine model systems. IgG isolated from the sera of adults immune to P. falciparum has been reported to act cooperatively with monocytes in in vitro parasite growth inhibition assays (20), suggesting that Abs may interact with Fc receptors on phagocytic cells to control parasitemia in vivo. In addition, in malaria-exposed individuals, IgG1 and IgG3, the two cytophilic isotypes in humans, predominate in protected subjects (29). Humans living in malaria endemic areas also have Abs that recognize multiple plasmodial Ags, and these Abs are often of the cytophilic isotypes in individuals with significantly reduced risk of malaria attacks or lower parasitemias and disease sequelae (30–36). However, these findings are not absolute, since levels of IgG1 have also been found to be higher in nonprotected subjects (37). In addition, other isotypes, such as IgM, have also been implicated in protection against P. falciparum in human subjects (38). In mice, protection against P. y. yoelii does not appear to be isotype specific. Both cytophilic and noncytophilic Abs are able to control parasitemia in this infection (8–10, 39), so that the isotype of protective Abs is not an important factor in humoral effector function.

In this study, anti-GST-PyC2 sera generated with RAS adjuvant (with a majority of IgG1, IgG2a, and IgG2b isotypes), alum-generated anti-GST-PyC2 sera (predominantly IgG1), mAb 302 ascites fluid (IgG3 isotype), and PyHIS (whose protective capacity is primarily mediated by IgG2a) (10) all were able to mediate protection against P. y. yoelii 17XL infections in mice. These protective effects were not limited to immunocompetent BALB/c mice but also functioned at equal efficiencies in FcγR γ-chain-deficient animals. Most of these samples contain Abs isotypes that would not be able to protect mice if their protective effects required ADCC, since the knockout mice are unable to perform ADCC with IgG1, IgG2a, and IgG2b Abs (26). Our data thus indicate that, in this
infection. Abs are able to control parasitaemia independently and do not require binding to Fc receptors. At present, it is not known how these Abs inhibit parasites, but they may act by blocking merozoite invasion (17) or may inhibit processing of MSP-1, which may be required for erythrocyte entry (40).

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