The Neutral Cysteine Proteinase of *Entamoeba histolytica* Degrades IgG and Prevents Its Binding

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Patients infected with *Entamoeba histolytica* generate specific IgG that does not prevent invasive amebiasis or recurrent infection. Studies investigated whether the effectiveness of the human humoral response was limited by cleavage of IgG by the extracellular neutral cysteine proteinase of *E. histolytica* trophozoites, one of the first amebic products to interact directly with components of host defenses. Purified proteinase cleaved polyclonal human and monoclonal murine IgG in a dose-dependent manner. Peptide sequencing of the major cleavage fragment(s), which contained the protein A binding site, suggested that cleavage occurred near the hinge region. Intact trophozoites also cleave IgG in both growth media and serum-free media. Cleaved monoclonal antibody to a 29-kDa surface antigen of *E. histolytica* bound to trophozoites 83.5% ± 6.7% less than did uncleaved antibody. These results suggest that cleavage of IgG by the extracellular cysteine proteinase may limit the effectiveness of the host humoral response.

Invasive amebiasis continues to be a major public health problem in developing countries, affecting >50 million people yearly. Although up to 10% of the world’s population is infected with *Entamoeba* species, <1% actually develop invasive disease. The large discrepancy between the number of infected patients and those with invasive amebiasis can be explained in part by the recent findings that there are two morphologically identical species: *Entamoeba histolytica*, which is capable of invasion, and *Entamoeba dispar*, which is not [1].

One virulence factor that may enable *E. histolytica* to invade is an extracellular neutral cysteine proteinase that degrades extracellular matrix and complement components [2, 3]. Cysteine proteinases in amebic lysates degrade IgA [4] and thus might also affect the efficacy of the humoral response by cleavage of IgG.

Specific IgG responses develop in >95% of patients with invasive amebiasis or even asymptomatic colonization with *E. histolytica* [5]. Despite this response, invasive amebiasis follows intestinal colonization, and recurrent amebic liver abscess, although rare, does occur. To determine if the systemic host immune response might be limited by proteolytic degradation of IgG, we investigated the interactions of polyclonal and monoclonal IgG with the purified neutral cysteine proteinase and live trophozoites.

Materials and Methods

*E. histolytica* trophozoites. Axenic *E. histolytica* (strain HM-1:IMSS; American Type Culture Collection, Rockville, MD) was grown in TYI-S-33 medium containing 15% bovine serum [1] and subcultured twice weekly. Amoebae were purified by chilling the flask, centrifuging the trophozoites at 300 g for 10 min, and washing three times in PBS.

Immunoglobulins. Human polyclonal IgG (Sigma, St. Louis) and monoclonal anti–bovine serum albumin (Sigma) were suspended at 1 mg/mL in PBS. Monoclonal antibody FP31 (IgG2a) was generated against a glutathione-S-transferase fusion protein of the 29-kDa thiol-rich surface antigen of *E. histolytica* as described [6]. Immunoglobulins were radio labeled with 125I to a specific activity of 106 cpm/μg of protein by the iodogen method [3].

Proteinase purification. Proteinase was purified from amebic trophozoites or secretions as described [2], and purity was confirmed on 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin [2] and silver-stained gels. The proteinase activity was determined by cleavage of the synthetic peptide, Boc-arginine-arginine-amine-7-methylcoumarin (Z-Arg-Arg-AMC; Enzyme System Products, Dublin, CA), as described previously [2, 3]. The activity of the proteinase was measured before each experiment.

Proteolytic digestion of IgG. Aliquots of unlabeled polyclonal IgG (2 μg) or radiolabeled murine monoclonal IgG (106 cpm added to 1 μg of cold IgG) were incubated for 1–18 h at 37°C with 0–50 U of purified proteinase/μg of IgG. Samples were boiled in the presence of the 10 μM specific inhibitor, P35017 (gift of Prototek, Dublin, CA), and reduced by adding an equal volume of 0.1 M Tris, 2.5% β-mercaptoethanol, and 2% SDS. Controls included samples in which the proteinase was inhibited with 10 μM P35017 for 30 min at room temperature before addition of IgG.

Samples were electrophoresed by SDS-PAGE (15% or 7.5%–15% gradient gels) under reducing conditions. Gels were dried and autoradiographed at −70°C on Kodak XAR-5 film. For immunoblots, gels were transferred to nitrocellulose, and membranes were blocked for 1 h with Tris-buffered saline (TBS) containing 1% nonfat milk and 0.5% Tween 20, washed twice in TBS/Tween, and incubated for 1.5 h with a 1:1000 dilution of alkaline phospha-
media were prepared for electrophoresis as detailed above. Min and washed three times with PBS. Aliquots of the pellets and counting. The percentage of binding was calculated as the counts seen in supernatant fractions. LC to remove excess unbound label, and the washes were pooled for fragment (F) from heavy chain (HC) is 7% 1
clonal antibody FP31 (1.34 µg) was incubated with amebic trophozoites was assessed by incubating 125I-labeled mono-
omoclonal antibody FP31 added to the amebic surface antigen was added. Controls included an unrelated monoclonal antibody to bovine serum albumin (Sigma).

Results

Dose-dependent cleavage of IgG. After incubation of polyclonal or monoclonal antibody with 10–50 U of proteinase/µg of protein, a major cleavage product of 30–35 kDa was detected (figure 1A). Cleavage was completely inhibited by preincubation with the specific cysteine proteinase inhibitor P35017 (data not shown). Similar results were obtained with polyclonal human IgG (figure 1B) and IgG1 and IgG2 subclasses of murine monoclonal antibodies (data not shown).

Localization of the cleavage site of IgG. To identify the specific site of cleavage, degradation products of human poly-
clonal IgG were probed with antibodies to protein A. After degradation of polyclonal IgG with 5–40 U of proteinase/µg of protein, Western blots of the cleavage products developed with alkaline phosphatase–conjugated protein A showed a single degradation product (figure 1B). When the cleavage product was transferred to polyvinylidene fluoride membranes for microsequencing, however, multiple sequences were obtained, suggesting that cleavage of the heavy chain occurred at more than one site. The major fragment contained the sequence X-Pro-Pro-X-Pro-Ala-Pro, which is located in the hinge region.

Cleavage of IgG by live trophozoites. To ensure that live trophozoites also cleaved IgG, 125I-labeled monoclonal antibody to the 29-kDa surface antigen was added to washed trophozoites in serum-free MEM-CH, in which >90% viability is maintained for 6 h [3], or amebic cultures in TYI-S-33. Autoradiographs of the resulting supernatants revealed a cleavage product of 30–35 kDa, similar to the cleavage product produced by purified proteinase (figure 2). Almost complete degradation of the heavy chain of IgG was detected in amebic pellets. Equivalent results were detected in amebic growth media (TYI-S-33), although incubation for at least 24 h was required (data not shown).

Binding of proteinase-cleaved IgG. To determine if proteinase-cleaved IgG could still bind to the amebic surface, we incubated purified amebae (2 x 10^5) in triplicate with cleaved

**Figure 1.** Dose-dependent cleavage of polyclonal and monoclonal IgG. A. Radiolabeled monoclonal antibody FP31 (10^6 cpm) was added to 1 µg of cold IgG and incubated with 0–50 U of proteinase/µg of IgG for 18 h at 37°C; cleavage products were visualized on autoradiographs of reduced polyacrylamide gels. Dose-dependent production of cleavage fragment (F) from heavy chain (HC) is seen. Light chain (LC) remains uncleaved. B. Western blot of proteinase-cleaved polyclonal IgG developed with protein A. Human polyclonal IgG (2.5 µg) was incubated for 18 h with 5–40 U of proteinase/µg of IgG. Resulting polyacrylamide gel was transferred to nitrocellulose and developed with alkaline phosphatase–labeled protein A. P = proteinase alone. HC and F are shown.

**Figure 2.** Cleavage of iodinated monoclonal antibody FP31 added to intact amebic trophozoites. After 4 h of incubation in serum-free Eagle MEM, HEPES buffer solution, ascorbic acid, and cysteine media, trophozoites were pelleted and extensively washed, and IgG fragments were detected on autoradiographs of pellets (P) and supernatants (S). C = control of antibody incubated in media alone. Similar cleavage fragment (F) from heavy chain (HC) is seen in supernatant fractions. LC = light chain.
or uncleaved radioactive monoclonal antibodies to the 29-kDa antigen. Binding by radiolabeled monoclonal antibody to bovine serum albumin was compared as a control. By 30 min, the cleaved antibody bound 83.5% ± 6.7% less to trophozoites than did uncleaved antibody (P < .0001). At 30 min, only 2.5% of added anti–bovine serum albumin was bound to the amebic pellet, likely representing nonspecific trapping.

Discussion

Invasion or even colonization by *E. histolytica* trophozoites stimulates both systemic and local antibody responses, in contrast to colonization with *E. dispar* [5]. Clearance of luminal parasites or protection afforded by systemic IgG has been difficult to demonstrate, however, and the level of antibody response correlates with the length of disease, not with the severity or clinical response to infection [5]. Similarly, protection of hamsters against liver abscesses does not correlate with the serum antibody response, although SCID mice were passively protected against infection by intraperitoneal injection of rabbit polyclonal immune serum [7]. The effect of passive antibody in the SCID mouse model may reflect both the quantity of antibody and the direct delivery of a bolus of antibody at the site of inoculation of the trophozoites.

The primary purpose of the amebic cysteine proteinase is most likely to break down endocytosed proteins. Significant quantities of cysteine proteinases are also released extracellularly [2] and thus are likely to be the first amebic products to interact with components of the host defense. The neutral cysteine proteinase has been shown to play a role in amebic invasion through degradation of components of the extracellular matrix, including collagen and elastin [2], activation of complement components by cleavage of C3 [5], and degradation of the anaphylatoxins, C3a and C5a [3]. Kelsall and Ravdin [4] concluded that cysteine proteinases were responsible for the degradation of IgA by amebic lysates on the basis of the effect of specific inhibitors. Cysteine proteinases are also a major virulence factor that differentiates *E. histolytica* from *E. dispar*. Clinical isolates of *E. histolytica* release significantly more cysteine proteinase activity and contain unique cysteine proteinase gene(s) [8]. If the cysteine proteinases of *E. histolytica* degrade IgG, this property may help explain the ineffectiveness of humoral defenses against amebiasis.

In the current studies, we showed that the purified amebic cysteine proteinase cleaved the heavy chain of both human polyclonal and murine monoclonal antibody in a dose-dependent manner (figure 1A). The major cleavage fragment of 30–35 kDa contained the protein A binding site (figure 1B). Microsequencing of the N-terminal fragment demonstrated, however, that several size-related fragments were produced. On the basis of the proline-rich sequence of one fragment, a region near the hinge was the major cleavage site.

To determine whether live amebic trophozoites could also cleave IgG, we investigated the cleavage of a radiolabeled monoclonal antibody to the 29-kDa thiol-rich surface antigen [5]. Trophozoites incubated with IgG in the serum-free media, MEM-CH [3], completely degraded the heavy chain in both supernatant and pellet fractions. The intensity of the light chain band was slightly decreased in the pellet fraction, most likely the result of internalization and subsequent degradation of bound immunoglobulin fragments by whole amebae [9].

Proteinase cleavage also prevented binding of a monoclonal antibody to an amebic surface antigen. The monoclonal antibody (FP31) against the 29-kDa surface antigen was pre-cleaved with 50 U of proteinase/μg of protein, resulting in >60% cleavage of the heavy chain. After 30 min of incubation with live trophozoites, binding of the cleaved antibody was reduced by 89% compared with intact antibody and was equivalent to the nonspecific binding of a monoclonal antibody to bovine serum albumin. Although capping and release of polyclonal antibody by *E. histolytica* trophozoites has been demonstrated [9], this phenomenon would not explain the significant difference in binding of cleaved and uncleaved IgG.

A number of potential human pathogens degrade immunoglobulins. The proteolytic digestion of IgA1 by mucosally invasive bacteria has been the best characterized [10]. These extracellular enzymes specifically cleave the heavy-chain hinge region of IgA1, and their presence is closely linked to pathogenicity of a number of bacteria [10]. Cysteine proteinases in amebic lysates have also been shown to cleave IgA1 [4].

Both protozoa and helminths have been shown to release proteinases that cleave IgG. *Trypanosoma cruzi* trypomastigotes bind nonimmune IgG through the Fab fragment and cleave the Fc fragment by the action of the major cysteine proteinase, cruzipain [11]. *Schistosoma mansoni* binds immunoglobulins by the Fc receptor and degrades the Fab portion into small peptides by the action of at least two proteinases [12]. Cysteine proteinases also play a role in the degradation of IgG by *Fasciola hepatica* [13], *Trichomonas vaginalis* [14], and *Tritrichomonas foetus* [15].

These data demonstrate that *E. histolytica* trophozoites are capable of cleaving bound and fluid-phase IgG. Cleavage of monoclonal antibodies specific to major surface antigens prevents their binding. Although in vivo studies would be required for definitive proof that cleavage of IgG by the amebic proteinase is a key mechanism of immune evasion, release of extracellular proteinases that cleave IgG is a common property of invasive parasites and may contribute in part to the failure of antibody alone to protect against amebic invasion.

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References

Molecular Analysis of Plasmodium vivax Relapses Using the MSP1 Molecule as a Genetic Marker

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Plasmodium vivax has hepatocytic dormant stages, hypnozoites, that cause relapses. This work compared paired isolates from primary attacks and relapses obtained from 10 individuals in Brazil using the merozoite surface protein 1 gene, PvMSP1, as a genetic marker. Four samples from primary attacks contained genetically mixed parasites harboring the 2 major PvMSP1 allelic forms. PCR revealed the presence of these 2 forms in the relapse parasites of 2 patients, demonstrating that the activation of hypnozoites is not clonal. DNA sequences from paired primary/relapse samples demonstrated that the parasites from the primary attack are identical to those in relapse samples in which the same allele forms were detected in both infections. Studies on the naturally acquired humoral immune responses of these patients against a recombinant protein expressing the C-terminus PvMSP1 demonstrated an increase in the titers, affinity maturation, and predominance of the IgG1 subclass during the relapse.

Plasmodium vivax is the most widely distributed human malaria parasite, causing ∼35 million cases annually [1]. In some parts of the world, including Brazil, where it reaches 70% of all the yearly malaria cases, this is the most prevalent species.

P. vivax malaria relapses [2] as clinical attacks that appear after schizonticidal treatment and that are partly responsible for the large socioeconomic burden of this human malaria. Unfortunately, the molecular basis of this phenomenon and its biologic significance remain elusive, mainly because of the difficulties in obtaining parasites from relapse cases.

One study has been published to date on the molecular analysis of paired primary and relapse isolates [3]. Significantly, it demonstrated that the strains causing the primary attack were not genetically different from those arising during relapses. In the present study, we compared paired primary and relapse parasites from 10 Brazilian patients by use of a polymorphic segment of the P. vivax merozoite surface protein 1 (PvMSP1) gene as the genetic marker. The naturally acquired IgG immune responses of these patients against a recombinant protein expressing the C-terminal region of PvMSP1 were also determined.