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Immunoglobulin M-Dependent Classical Complement Pathway Activation in Killing of *Pentatrichomonas hominis*

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Complement pathway activity in the killing of *Pentatrichomonas hominis* was investigated in this study. At 10^5 organisms per ml, *P. hominis* was completely killed by the presence of 1% normal human serum. In contrast, no killing effect on *P. hominis* was observed when specific antibodies were absorbed or when the complement was destroyed. Moreover, Mg^{2+}-ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid-treated serum had no killing effect on *P. hominis*, while serum heated at 50°C or treated with zymosan killed *P. hominis* as well as did normal human serum. Further study using gel filtration (Sephacryl S-300) and affinity chromatography (protein A) revealed that immunoglobulin M (IgM; 20 μg/ml) alone was responsible for the complement activation in the killing of *P. hominis*, but both IgA (24 μg/ml) and IgG (180 μg/ml) had no effect on complement-mediated lysis. On the other hand, IgG at 1,260 μg/ml completely inhibited complement-mediated killing by IgM, suggesting that a blocking factor is present in IgG. The results of this study indicate that a mechanism of IgM-dependent classical complement pathway activation contributes to the killing effect of normal human serum on *P. hominis*.

Pentatrichomonas hominis inhabits the large intestines of humans and many other species of mammals. In less-developed parts of the world, this organism is commonly found in diarrheal stool and has been associated with abdominal discomfort (10), although it has not been shown to be pathogenic for humans. There is also no convincing evidence that *P. hominis* can cause disease in immunocompromised patients, but there could be a potential problem. In fact, other intestinal protozoa, such as *Cryptosporidium* sp. and *Isospora* sp. were considered innocuous until the acquired immune deficiency syndrome appeared. Moreover, several reports have aroused suspicion that this commensal organism could be found in areas other than the gastrointestinal tract (13, 24, 26). A trichomonad species which could have been *P. hominis* was found in the cerebrospinal fluid of a patient with esophageal carcinoma, and spread from the gut was considered possible (13). We also reported a case of *P. hominis* infection in a lymphoma patient with severe diarrhea (23). With the increased incidence of opportunistic infections in immunocompromised and immunodeficient hosts, the pathogenesis of *P. hominis* infection could become an important subject for investigation. Little work on the immune response to *P. hominis* has been undertaken. To elucidate the role of humoral immunity in the defense mechanisms against *P. hominis*, complement pathway activity in the killing of this organism was intensively investigated in this study.

MATERIALS AND METHODS

Organisms. Axenically cultivated *P. hominis* (NDMC), originally isolated from a lymphoma patient in our laboratory (23), was maintained in a modified medium identical to the TYI-S-33 medium of Diamond et al. (4), with the exception that 0.5% Panmede was added. Two additional strains, obtained from the American Type Culture Collection, were ATCC 30000 and ATCC 30098. Other organisms, including bacteria used for serum absorption, were *Trichomonas vaginalis, Giardia lamblia, Salmonella enteritidis,* and *Escherichia coli.*

Counting of protozoa. The number of flagellates per culture was determined with a Coulter counter (model D industrial; Coulter Electronics, Inc., Hialeah, Fla.) with a 70-μm aperture tube.

Sera. Venous blood from 24 healthy donors who had no recent history of diarrhea or medication within the past 6 months was allowed to clot at room temperature and then centrifuged at 400 × *g*. C-reactive protein and rheumatoid factor were determined with a nephelometer (Auto ICS; Beckman Instruments, Inc., Fullerton, Calif.), and only test-negative sera were included in this study. Because no cysts are formed, *P. hominis* infection in healthy donors has been ruled out by the absence of trophozoites in six consecutive stool examinations. Serum from each donor was tested for the killing effect on *P. hominis* individually. These normal human sera were also pooled and stored at −70°C in 1-ml aliquots. Sera from premature (n = 6) and newborn (n = 7) infants were obtained through the courtesy of K. D. Yang, Department of Pediatrics, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan. Heat inactivation to abolish serum complement activity was performed at 56°C for 30 min. Specific antibodies were removed by absorption with homologous organisms at 5×10^8 flagellates per ml of serum at 4°C for 6 h or at 10^{10} bacteria per ml of serum at 4°C for 1 h to produce various absorbed sera. Before use, all sera, both treated and untreated, were assayed for antibody reactive to *P. hominis* by means of agglutination and an indirect immunofluorescence assay as described below. To study the activity of the classical complement pathway (CCP), the serum was depleted of the alternative complement pathway (ACP) by treatment with zymosan A (4 mg/ml of serum; Sigma Chemical Co., St. Louis, Mo.) (1, 18) or depleted of factor B by heat treatment at 50°C for 20 min (2). To deplete CCP activity while leaving the ACP intact, chelation of Ca^{2+} in normal human serum and *P. hominis*-absorbed serum was performed by addition of 10 mM ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',*

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N'-tetraacetic acid (EGTA; Sigma) containing 1 mM $MgCl_2$ to the sera (5).

Complement assay. Hemolytic complement activity was measured with antibody-sensitized sheep erythrocytes (CH_{50} for CCP) (14) or unsensitized rabbit erythrocytes (AP_{50} for ACP) (19). *P. hominis*-absorbed serum retained normal ACP activity (284 ± 37 U/ml [mean ± standard deviation]; $n = 6$) and CCP activity (157 ± 19 U/ml). For CCP-inactivated serum, CCP activity was 0 and ACP activity was 271 ± 29 U/ml. For ACP-depleted serum, CCP activity was 196 ± 21 U/ml and ACP activity was unmeasurable.

Immunoglobulin preparation. Immunoglobulin M (IgM)-intact, but IgG-depleted serum (hereafter called the IgM fraction) was prepared by gel filtration on Sephacryl S-300 followed by affinity chromatography on Sepharose-protein A (Pharmacia, Uppsala, Sweden) as described by Vidal and Conde (25). After extensive washing of the protein A column with phosphate-buffered saline (PBS), IgG was eluted with 0.1 M citrate buffer, pH 2.7. Serum IgA was purified by salt-mediated hydrophobic chromatography on L-phenylalanine-conjugated Sepharose 4B (Pharmacia). IgM, IgG, and IgA fractions were concentrated to their respective physiological concentrations with the aid of an Ultrafiltration Cell (model 8050; Amicon Corp., Danvers, Mass.). Complement in the IgM, IgG, and IgA fractions were inactivated (56°C for 30 min), and samples of the preparations were stored at −70°C. The concentrations of IgM, IgG, and IgA in the three fractions were measured by radial immunodiffusion with commercially available kits (Behringwerke, Marburg, Federal Republic of Germany). These analysis revealed that 70 to 90% of the IgG, 60 to 80% of the IgM, and 30 to 40% of the IgA were recovered from normal human serum.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified immunoglobulins were dissolved in a sample buffer that contained 0.1 M Tris hydrochloride, 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, and 5% mercaptoethanol and boiled for 3 min. Samples were electrophoresed in a discontinuous 0.1% sodium dodecyl sulfate–12% polyacrylamide gel on a Minigel apparatus (Bio-Rad Laboratories, Richmond, Calif.) with the buffers of Laemmli (12) to reduce the sample amount needed. Approximate molecular weights were estimated by using standard proteins. Some gels stained with Coomassie blue were scanned by densitometry.

Indirect fluorescent-antibody test. Acetone-fixed trophozoites (10^4) were incubated with 10 μl of various treated sera (1:10) for 30 min at 20°C. After three washings in PBS, the organisms were incubated with 10 μl of a 1:40 dilution of fluorescein-conjugated goat anti-human C3, anti-human IgG, anti-human IgA, and anti-human IgM (Organon Teknika, Malvern, Pa.), respectively, for 30 min at 20°C in the dark. The parasites were then washed three times in PBS, mounted on microscope slides, and examined with a Zeiss fluorescence microscope.

Quantitative indirect immunofluorescence assay. Formalin-fixed trophozoites (10^5/ml) were treated with different concentrations of IgG in the presence of 1% normal human serum at 37°C for 1 h. Opsonized trophozoites were washed and suspended in 1 ml of PBS. Each milliliter of opsonized trophozoites was reacted with 100 μl of 1:4 diluted fluorescein-conjugated goat anti-human IgM and IgG (Organon Teknika), respectively, for 30 min at 20°C in the dark. The parasite suspensions were washed three times and suspended to 1 ml in PBS. The fluorescence of the secondary antibodies attached to the parasite surface was quantitatively detected with a fluorimeter (LS-2B filter; Perkin-Elmer, Beaconsfield, England) set at wavelengths of 485 and 525 nm. The results were expressed in arbitrary units.

Agglutination test. An agglutination test was used to determine the titers of all of the sera. This consisted of preparing serial twofold dilutions of serum in Hanks balanced salt solution and laying 96-well U-bottom plates (100 μl per well in triplicate). One hundred microliters of a parasite suspension containing 2×10^4 *P. hominis* was added to each well. The contents of the wells were mixed, and the plates were incubated at 37°C for 1 h. Agglutination was determined by microscopic examination with an inverted microscope. Hanks balanced salt solution was used as a control.

Serum-killing assay. Duplicate reaction mixtures were set up containing 10^5 *P. hominis* per ml which were incubated with various serum preparations at 37°C and rotated end over end for 1 h. Percent parasite survival with this procedure was determined by a regrowth assay performed as previously described (8). The generation time for *P. hominis* is 4.4 ± 0.4 h ($n = 3$). In brief, 100 μl of each incubation sample was added in duplicate to 10 ml of freshly prepared TYI-S-33 medium in screw-cap culture tubes (16 by 125 mm). After 48 h of incubation, the concentration of parasites in each culture tube was determined by counting in a Coulter counter. The initial number of viable parasites was then obtained by extrapolation from the standard reference growth curve by using the total number counted at 48 h. The establishment of this assay was based on the fact that the growth of the remaining viable parasites was not inhibited, which was confirmed by prolonged culture and observation for up to 2 weeks. Finally, the percentage of trophozoites surviving the serum treatment was calculated by the following formula: Viability index = number of viable parasites following serum treatment as determined from a standard reference growth curve/total number of parasites incubated with each serum preparation.

RESULTS

Effect of normal human serum on *P. hominis* killing. The basic experiments on the serum killing of three strains of *P. hominis* were performed with different serum concentrations (0.1, 1, and 5%) and at three reaction time intervals (10, 20, and 30 min). At 10^5/ml, the organisms were totally lysed by 1% normal human serum during 30 min of incubation. No killing of *P. hominis* could be seen when the concentration of normal human serum was 0.1%. Therefore, 1% serum was used in this series of experiments. Each of 24 serum samples had the same capacity to kill *P. hominis*; i.e., *P. hominis* was completely lysed by each normal human serum at 1%. However, of the premature ($n = 6$) and newborn ($n = 7$) sera tested, none was found to have a killing effect on this parasite.

Measurement of serum antibodies against *P. hominis*. IgM, IgG, and IgA isotypes of serum antibodies were detected in normal human serum by immunofluorescence with heavy-chain-specific antisera as described above. In each instance, a diffuse pattern of staining was observed. Purified individual immunoglobulin (IgM, IgG, and IgA) reacted by indirect fluorescent-antibody test with *P. hominis* also revealed the same patterns (data not shown). All 24 sera agglutinated *P. hominis*. Agglutination tests revealed that heat-inactivated pooled serum had a titer of 256. Following absorption with *P. hominis*, the titer was reduced to 4. Absorption with other protozoa (*G. lamblia*, etc.) failed to remove anti-*P. hominis*

TABLE 1. Viability index for *P. hominis* in the presence of different serum treatments

Serum	Mean ± SD % viability[a]
Normal human serum	0
P. hominis-absorbed serum	108 ± 19
Heat-inactivated serum (56°C, 30 min)	113 ± 14
P. hominis-absorbed serum–heat-inactivated serum	0
None (Hanks balanced salt solution [control])	105 ± 11
Normal human serum treated with Mg^{2+}-EGTA	109 ± 17
Normal human serum treated with zymosan	0
Normal human serum heated at 50°C	0

[a] Percent viability is the mean of determinations made in nine separate experiments.

antibody from the sera. Hanks balanced salt solution was used as a control, and no spontaneous agglutination was observed.

Killing effects of serum components on *P. hominis*. Although normal human serum appeared to kill *P. hominis*, neither *P. hominis*-absorbed serum nor heat-inactivated serum had such an effect on the parasite. However, the lytic effect on *P. hominis* was restored when both *P. hominis*-absorbed serum and heat-inactivated serum were present (Table 1), suggesting that both complement and antibody are responsible for the ability of normal human serum to kill *P. hominis*.

Effects of various absorbed sera on *P. hominis* survival. Serum absorbed with individual strains of *P. hominis* failed to kill either the homologous or heterologous strains of *P. hominis* (Table 2), suggesting that common antigens exist among these strains of *P. hominis*. On the other hand, serum absorbed with other species of protozoa (*T. vaginalis* and *G. lamblia*) or bacteria (*S. enteritidis* and *E. coli*) remained lethal for *P. hominis* (Table 2), suggesting that these organisms lacked a common surface antigen present in *P. hominis*.

Effects of immunoglobulin fractions on *P. hominis* survival. The effects of immunoglobulin fractions on the viability of *P. hominis* were tested with or without complement. Without complement, immunoglobulin fractions, either individually or in combinations, did not kill *P. hominis*. With *P. hominis*-absorbed serum (as the complement source), only the IgM fraction from either individual or pooled sera was capable of killing the parasite (Table 3). IgM fraction purity was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis, which revealed only two single bands with molecular weights of 65,000 (heavy chain) and 23,000 (light chain), respectively (data not shown). IgM purified from myeloma (Jackson Immuno Research Laboratories Inc., West Grove, Pa.) was used for comparison and was found to be able to activate complement leading to *P. hominis* lysis. Since IgG and IgA antibodies were able to bind with *P. hominis*, as shown by immunofluorescence, we attempted to determine whether these two classes of immunoglobulins could interfere with IgM-dependent, complement-mediated lysis of the parasite. IgG at 540 μg/ml significantly ($P < 0.01$) reduced *P. hominis* killing by normal human serum. At 1,260 μg/ml, it completely inhibited the killing activity of normal human serum (Fig. 1). To determine whether high concentrations of IgG actually inhibited IgM binding, a quantitative indirect immunofluorescence assay was performed. Binding of IgM to *P. hominis* was inhibited by increased concentrations of IgG (Fig. 2). These results suggest that a blocking factor is present in IgG. In contrast, IgA did not block the killing activity of normal human serum (data not shown). A complement hemolytic assay was performed to further examine whether the additional IgG fraction could interfere with complement activity in normal human serum. Both complement pathway activities in the hemolytic assay did not change in the presence of additional IgG (data not shown).

TABLE 2. Viability index for *P. hominis* in the presence of different absorbed sera at 1%

Serum	Mean ± SD % viability[a]
P. hominis (NDMC)-absorbed serum	104 ± 11
P. hominis (ATCC 30000)-absorbed serum	110 ± 9
P. hominis (ATCC 30098)-absorbed serum	107 ± 8
T. vaginalis (ATCC 30238)-absorbed serum	0
G. lamblia (NDMC)-absorbed serum	0
S. enteritidis (NCTC 6676)-absorbed serum	0
E. coli (O7 K1)-absorbed serum	0

[a] Percent viability is the mean of determinations made in three separate experiments.

TABLE 3. Effects of immunoglobulin fractions on *P. hominis* viability

Immunoglobulin fraction(s)	Mean ± SD % viability[a]	
	Without complement	With complement
IgM	107 ± 6	1 ± 1
IgA	119 ± 12	109 ± 13
IgG	114 ± 9	116 ± 12
IgM-IgA	108 ± 11	2 ± 1
IgM-IgG	121 ± 17	3 ± 2
IgA-IgG	108 ± 10	119 ± 16
IgM-IgA-IgG	116 ± 18	2 ± 2

[a] Percent viability is the mean of determinations made in six separate experiments. *P. hominis*-absorbed serum was used as the source of complement.

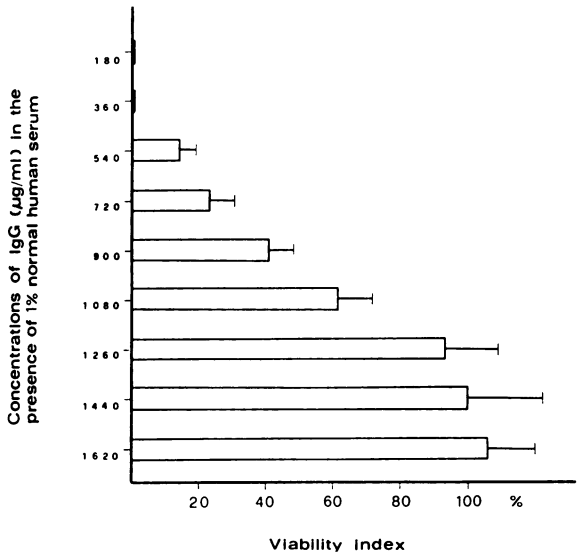

FIG. 1. Effect of IgG on *P. hominis* killing by normal human serum. IgG at 540 μg/ml interfered with killing by normal human serum ($P < 0.01$; $n = 6$), while at 1,260 μg/ml it completely inhibited killing by normal human serum.

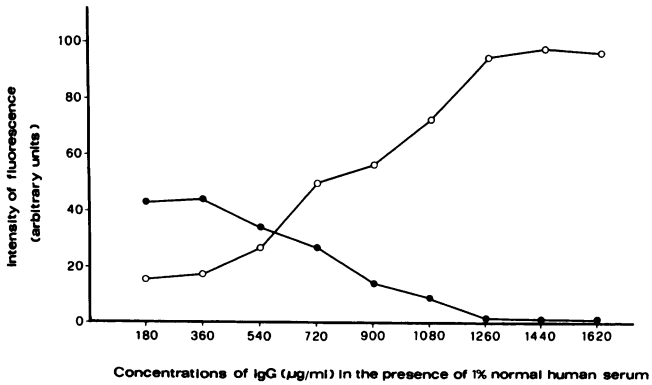

FIG. 2. High concentrations of IgG inhibiting the binding of IgM to *P. hominis* determined by indirect immunofluorescence assay. Fluorescein-conjugated anti-human IgM (●) was hardly detectable on *P. hominis* when the concentration of IgG was up to 1,260 μg/ml, in which fluorescein-conjugated anti-human IgG (○) bound *P. hominis* maximally. The symbols represent the means of triplicate values.

Complement pathway activity in *P. hominis* killing. Whereas C3 was detected by indirect immunofluorescence on parasites reacted with normal human serum, the parasites failed to stain following incubation in *P. hominis*-absorbed serum (data not shown), providing further evidence that complement-fixing antibodies are necessary for complement activation. Normal human serum treated with Mg^{2+}-EGTA did not kill *P. hominis*, while ACP-depleted sera killed the organisms as well as did normal human serum. This indicates that CCP activity exclusively contributes to serum killing. The antibody involved in activation of the CCP leading to *P. hominis* lysis is IgM (Table 4).

DISCUSSION

While it is recognized that *P. hominis* inhabits intestines, where it encounters the mucosal immune system, we chose to study the ability of low concentrations of normal human serum to kill this parasite to determine whether complement and antibody participate in the lethal event. Accordingly, a system was developed to quantitate parasite survival following exposure to serum. Our results indicate that *P. hominis* was killed by normal human serum through a mechanism of IgM-dependent classical pathway activation.

TABLE 4. Complement pathway activity in *P. hominis* killing

Serum	Mean ± SD % viability[a]
PAS[b]	107 ± 19
PAS treated with Mg^{2+}-EGTA	111 ± 15
PAS treated with Mg^{2+}-EGTA-IgM	108 ± 11
PAS treated with Mg^{2+}-EGTA-IgA	107 ± 9
PAS treated with Mg^{2+}-EGTA-IgG	114 ± 14
PAS treated with zymosan	104 ± 12
PAS treated with zymosan-IgM	3 ± 2
PAS treated with zymosan-IgA	109 ± 16
PAS treated with zymosan-IgG	114 ± 10
PAS heated at 50°C	107 ± 11
PAS heated at 50°C-IgM	4 ± 4
PAS heated at 50°C-IgA	121 ± 17
PAS heated at 50°C-IgG	119 ± 21
Heat-inactivated serum (56°C, 30 min)	111 ± 8
PAS–heat-inactivated serum	0
Normal human serum	0

[a] Percent viability is the mean of determinations made in six separate experiments.
[b] PAS, *P. hominis*-absorbed serum.

The observation that heat-inactivated serum and *P. hominis*-absorbed serum failed to kill *P. hominis* strongly indicated the participation of both complement and antibody in the killing event. We found that chelation of serum with EGTA, which is known to block complement activation by the classical pathway, abrogated the lethal activity of serum. These findings strongly suggest that killing is mediated by activation of the classical pathway via antibody reacting with parasite antigens. This deduction is supported by the deposition of C3 on the parasite and the fact that ACP-depleted serum was capable of killing *P. hominis*.

Complement activity in the killing of parasitic protozoa has been investigated intensively. *Leishmania donovani* promastigotes (17), *Toxoplasma gondii* (22), and *G. lamblia* (7) were killed by normal human serum through CCP activation, while *Trichomonas vaginalis* (6), *Entamoeba histolytica* (11, 16), and *Naegleria fowleri* (9, 27) activated complement via the alternative pathway. More recently, it has been emphasized that activation of the alternative pathway is important in the lysis of nonpathogenic strains of *E. histolytica* (20). *P. hominis* has never been shown to be pathogenic. However, the inability of *P. hominis* to activate the ACP was not clear. Complement activation could be strongly dependent on the nature of the parasite surface. Therefore, immunochemical studies on the membrane of this parasite are under way.

In a study of the mechanism of killing of *G. lamblia* trophozoites by complement, Deguchi et al. used a two-stage procedure and demonstrated that activation of the alternative pathway could be triggered by the initial activation of C1 (3). In our study, *P. hominis* was exposed to human serum in a reaction mixture without washing; therefore, complement activation occurred in a more natural way. Although we could not exclude the possibility that a unique complement pathway that requires C1 and factor B is involved in the killing of *P. hominis*, the present study clearly showed that factor B-depleted serum did not decrease the capacity to kill this parasite, suggesting that the role of alternative pathway activation in the lysis of *P. hominis* is not crucial.

Both normal human serum and heat-inactivated serum contained antibodies reactive with three strains of *P. hominis*, as determined by indirect immunofluorescence and agglutination tests. Absorption of the serum with unrelated protozoa and bacteria failed to reduce the agglutination titer against the homologous parasites, indicating that the antigenic epitopes involved in the agglutination of *P. hominis* are parasite specific. When normal human serum was absorbed with the same parasite preparations and tested for killing activity, similar findings were obtained; i.e., absorption with *P. hominis* depleted the killing activity of normal human serum, whereas absorption with other protozoa and bacteria failed to do so.

Fractionation of serum into isotype-enriched fractions demonstrated that the immunoglobulin isotype responsible for activation of the complement system was IgM. That IgG-enriched fractions of normal human serum blocked the *P. hominis*-killing activity of normal human serum was a surprising but significant finding. It was shown that the IgG fraction added to normal human serum was not anticomplementary. The data suggest that antibodies in the IgG fraction, although capable of binding to the parasite, failed to activate complement. While the identity of the subclass of

the IgG antibody blocking the killing event remains to be determined, it is well known that IgG_4 is incapable of fixing complement. It has been proposed that natural IgG binds to certain gonococci possessing the appropriate receptor, enabling them to disseminate in the blood by protecting them from being killed by antibody and complement (15). Further efforts are warranted to determine whether IgG plays a role in the extraintestinal spread of *P. hominis*.

The question arises as to the origin of these parasite-specific antibodies in the normal human serum used in these studies. Natural antibodies, including IgM, have been shown to be major immunoglobulins involved in the complement-mediated lysis of leishmanias (17, 21). IgM purified from myeloma (Jackson), compared with the IgM fraction purified in this study, had a similar ability to activate complement, leading to *P. hominis* lysis. Therefore, it is possible that the presence of these IgM antibodies is due to previous inapparent infection or that they are naturally occurring antibodies which result from stimulation by some organisms within the environment.

The fact that *P. hominis* is killed by complement may have nothing to do with its lack of pathogenicity in humans. However, the ability of low concentrations of serum to kill this parasite not only prevents multiplication of the parasites but also blocks invasion of the submucosa by the parasites in individuals with complement-fixing antibodies. Perhaps this is one of the most effective defense mechanisms to counteract this parasite infection.

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