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Loss of susceptibility to complement lysis in *Entamoeba histolytica* HM1 by treatment with human serum

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SUMMARY

Entamoeba histolytica HM1, exposed to a series of treatment with normal human serum (NHS), progressively lost suscetibility to complement lysis. Trophozoites were incubated daily with unheated or heat-inactivated NHS for 2 hr at 36°, starting with 10% v/v serum and increasing by 5% every 3 days up to 40% NHS. Resistance to complement lysis was also obtained with two different HM1 clones but not with the low virulent strain HK9. Induction of resistance dependent on the number of NHS treatments, with a maximal 50% reduction occurring after 11 treatments. Susceptibility to complement-dependent lysis was regained 6 weeks after serum treatments were terminated, suggesting that resistance to lysis was an acquired rather than a genetic property.

INTRODUCTION

Survival of trophozoites of *E. histolytica* in human tissues, in the face of nature host defences and an active immune response (Maddison, Kagan & Elsdon-Dew, 1968; Krupp & Powell, 1971; Sepulveda & Martinez-Palomo, 1982), may be the result of both virulence determinants of the parasite and host factors. Studies on *E. histolytica* strains displaying varying degrees of virulence have defined some factors related to their pathogenic activity (Sargeaunt & Williams, 1979; Martinez-Palomo, 1982; Orozco *et al.*, 1983; Muñoz *et al.*, 1984).

It is now recognized that the complement system plays a significant role in host defence against *E. histolytica*. Lysis of trophozoites can result from complement activation in either the presence or absence of specific antibodies (Ortiz-Ortiz *et al.*, 1978; Huldt *et al.*, 1979; Calderon & Schreiber, 1985). In order to understand how amoebic infection arises despite the lytic activity of complement, we examined whether trophozoites that were repeatedly exposed to normal human serum (NHS) displayed an altered susceptibility to C-killing. Our results showed that serum-treated amoebas progressively developed a significant resistance to complement-mediated lysis. This resistance was reversible since the amoebas regained their complement sensitivity after termination of the serum treatments.

Abbreviations: C, complement; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; NHS, normal human serum; TYI-S-33 medium, trypticase yeast extract iron serum; VBS-A, veronal-buffered saline for amoebas.

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MATERIALS AND METHODS

Entamoeba cultures

E. histolytica HK9: IMSS and HM1:IMSS (obtained from Margarita de la Torre, Centro de Estudios sobre Amibiasis, IMSS, Mexico) were cultured in TYI-S-33 medium (Diamond, Harlow & Cunnick, 1978). Most assays for susceptibility to lysis and treatments with complement were carried out with trophozoites during the logarithmic phase of growth.

Modified TYI-S-33 medium for complement lysis

One litre of solution in deionized water contained the following reagents: Biosate (BBL), 30 g; glucose, 10 g; NaCl, 2 g; HEPES (Calbiochem, La Jolla, CA, Cat. No. 391338), 10 mM pH 7·2; L (+) ascorbic acid, 200 mg; ferric ammonium citrate, brown powder (Sigma, St Louis, MO, F-5879), 23 mg; $MgCl_2$, 0·5 mM, and $CaCl_2$, 0·15 mM. The solution was sterilized at 121° for 15 min.

Human complement (NHS)

Human serum from normal donors, negative in anti-*E. histolytica* HM1 antibodies by ELISA (Engvall, Jonsson & Perelmann, 1971), in individual lots of 100 or 200 ml, was distributed in 5 ml aliquots and stored at −70°. Each lot was used as source of complement to assay trophozoite susceptibility to lysis and for complement treatments of amoebas to induce resistance to complement damage.

^{32}P-labelling of trophozoites and specific isotope release from lysed cells

The medium used for labelling was the TYI-S-33 with 4% v/v calf serum but without the addition of phosphate salts. Amoebas at 8×10^4/ml were incubated with 5 μCi/Ml of ^{32}P-labelled

orthophosphate (New England Nuclear, Boston, MA) for 16 hr at 37°. Cells were washed three times with VBS-A and were resuspended to a density of 2×10^4/ml. Incorporation of radioactivity was 2·4% of total with a specific activity of 330,000 c.p.m./10^5 trophzoites. Complement ability to lyse labelled amoebas was performed in triplicate tubes in a total volume of 1 ml with 2×10^4 microorganisms in the presence of NHS or heat-inactivated NHS during 30 min at 37°. Isotope release was assessed in 0·5 ml supernatant and mixed with 5 ml Insta-gel for analysis of radioactivity. Specific ^{32}P release was calculated as follows:

$$(A - B/C - B) \times 100$$

in which A and B are the d.p.m. released from amoebas in the presence of NHS or heat-inactivated NHS at same condition, respectively, and C is the amount of d.p.m. released from same number of trophozoites frozen and thawed three times.

Quantification of lysis of E. histolytica

In polypropylene conical tubes, 0·2 ml aliquots of trophozoites at $1{\cdot}5 \times 10^6$/ml were mixed with 0·2 ml of active or heat-inactivated NHS at indicated concentrations in cold veronal-buffered saline for amoebas (VBS-A, veronal buffer containing 5 mM diethylbarbiturate, 0·5 mM $MgCl_2$, 0·15 mM $CaCl_2$ and 193 mM NaCl, pH 7·2). The mixtures were incubated at 36° for 30 min, resuspending cells every 8 min. Samples were centrifuged for 3 seconds at 10,000 *g* to remove serum. Residual trophozoites were resuspended in 0·25 ml culture medium to count viable cells by phase contrast in an improved Neubauer chamber. Percentage of lysis was defined as the decrease in viable amoebas in NHS as compared to the control with heat-inactivated serum calculated as follows:

$$\frac{(\text{viable in control} - \text{viable in NHS})}{\text{viable in control}} \times 100.$$

Susceptibility to lysis under equal complement concentrations in lytic assay was defined as the quotient of:

$$\frac{\%\text{ lysis in NHS pretreated amoebas}}{\%\text{ lysis in inactive NHS pretreated amoebas}} \times 100.$$

Cloning of E. histolytica *HM1*

We followed the method of Gillin & Diamond (1978), but with Seaplaque Agarose (Marine Colloids Div. HMC Corp., Rockland, ME) instead of Bacto Agar because it gelifies at a lower temperature. One-hundred μl containing 100 trophozoites were mixed with 30 ml of 0·32% w/v Seaplaque Agarose in TYI-S-33 at 37° in a 30 ml Falcon flask. The flasks were first incubated in ice-water for 10 min to allow for gelification, and then in a water-bath at 37° for 15 min before culturing. Colonies formed from a single cell were collected with a long tip pipette and placed in liquid culture for 10 days. These amoebas were then recloned four consecutive times and clones $5C_1$ and $5C_2$ obtained.

Complement treatments of E. histolytica

Trophozoites, 1×10^6, were treated daily with 4 ml of active or heat-inactivated NHS as control at different concentrations in modified TYI-S-33 medium for 2 hr at 36°. After each serum treatment, parasites surviving complement lysis, approximately $2{\cdot}5 \times 10^5$, and an equal number of cells from inactivated NHS condition were washed and grown in normal TYI-S-33 medium for 20 hr to repeat lytic treatment. Because of the high susceptibility of amoebas to complement effect, low serum doses were utilized for initial exposures, starting with 10% v/v for three treatments and continuing with 5% increments after three to five treatments, as amoebas showed increased resistance to complement damage, up to a maximum of 40% serum which lysed 95% cells in the control and 35–50% in the NHS-treated population.

RESULTS

Lysis and solubilization of trophozoites by complement

In order to examine the effect of complement on amoebas, the organisms were incubated in 20, 30, 40 and 50% untreated and heat-inactivated NHS at 56° for 30 min. At ranges of 20–40% NHS, two-thirds of the lysed amoebas disintegrated and were therefore not observed in the microscopic field. Thus, in the presence of 30% heat-inactivated NHS (unlysed control) 110 ± 10 trohozoites were detected in a 1 mm^2 area; eight appeared dim (dead) while the remainder were refractible with a defined membrane and were considered viable. With 30% untreated NHS only 38 amoebas were observed (35% of control), of which 16 trophozoites were viable and 22 non-viable since they appeared dim and displayed different degrees of membrane disruption.

In order to demonstrate that counting viable amoebas with refractile plasma membranes was an objective method of determining the percentage of trophozoite lysis by complement, we compared the specific release of ^{32}P from ^{32}P-labelled amoebas with the microscopic evaluation. Results from both methods were identical (Fig. 1). Therefore, we used the cell counting method throughout the remainder of the study.

Susceptibility to complement lysis is decreased in *E. histolytica* HM1, not in HK9 strain, followed treatment with NHS

Figure 2 demonstrates that trophozoites of *E. histolytica* HM1 and a HM1 clone ($5C_1$) treated up to seven times with NHS displayed reduced susceptibility to complement lysis. Induction of resistance was dependent on the number of NHS treatments.

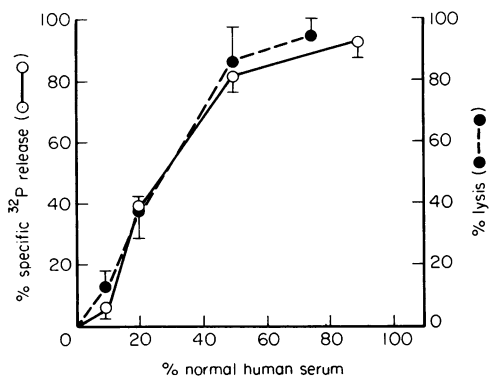

Figure 1. Comparable quantitative measurements of specific release of ^{32}P and microscopic evaluation (percentage of lysis) of NHS damage on *E. histolytica* trophozoites.

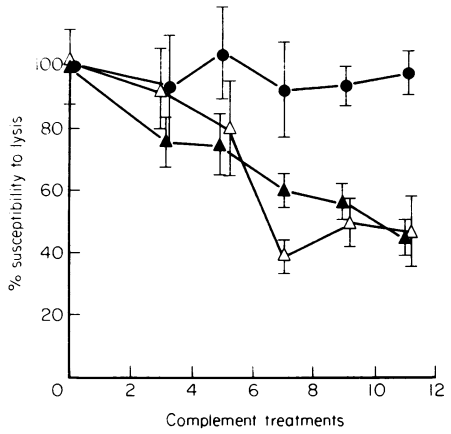

Figure 2. Dependency on NHS treatments of susceptibility reduction to complement lysis in *E. histolytica* HM1. Amoebas were treated with 15, 20 and 25% active or heat-inactivated NHS, three and five times, respectively, as indicated in the Materials and Methods. Susceptibility to lysis was determined with NHS concentrations equivalent to the amount used in experimental treatment. *E. histolytica* HK9 (●——●), HM1 (▲——▲), and its derived clone $5C_1$ (△——△).

Table 1. Reduction of susceptibility to complement lysis in clones of *E. histolytica* HM1 pretreated with NHS

	% lysis		
	Serum regimens*		
Lytic assay†	Untreated control	Inactivated NHS control	NHS
25% NHS			
Uncloned HM1	35±6	44±6	15±7
Clone $5C_1$	55±15	45±6	13±3
Clone $5C_2$	44±5	60±5	16±4
40% NHS			
Uncloned HM1	86±7	91±5	51±10
Clone $5C_1$	85±10	88±6	43±4
Clone $5C_2$	92±5	98±7	60±6

* Cloned trophozoites were treated three times with each of the following concentrations: 20, 30 and 40% active and inactive NHS.

† Lysis was assayed with 0, 25 and 40% NHS for 30 min at 36°. Data represent the mean±SD ($n=6$); $P<0{\cdot}01$, at Table IV of Fisher & Yates (1949), testing NHS regimen with either control.

Treatment with heat-inactivated NHS failed to induce the resistant phenotype. No alteration in complement susceptibility could be induced in Strain HK9, even following 50 treatments with NHS.

Induction of resistance to complement in cloned trophozoites of *E. histolytica* HM1

We next determined whether complement sensitivity could be altered in cloned trophozoites. Two different clones were used that had been derived by five consecutive cloning in semisolid agarose.

Both clones were first treated nine times with either NHS or inactivated NHS as described in the Materials and Methods. Table 1 shows that, like uncloned populations, pretreatment of the trophozoite clones with NHS resulted in cultures with decreased susceptibility to lysis. Even at higher serum concentrations (40%), where cell damage was more extensive, complement-conditioned amoebas displayed a significant resistance: a 40% decrease in lysis ($P<0{\cdot}005$). Thus, resistance to lysis appears to be an acquired trait and not a result of selecting a genetically resistant subclone of *E. histolytica*.

In an attempt to increase resistance to C-lysis further, cloned trophozoites under 40% NHS regimens, as indicated in Table 1, received 64 additional exposures to 40% NHS. Susceptibility to C-lysis of this population is depicted in Fig. 3. Complement-adapted parasites showed a significant degree of resistance to C-lysis at different serum concentrations, as compared with the control. Thus, C-pretreated amoebas, incubated with 30, 40 and 60% NHS, were lysed only 18, 36 and 57%, as well as the control population incubated with the respective serum concentration. Table 2 shows cell numbers obtained after 5 hr and 29 hr in a culture of amoebas that had been exposed to 25% C for 2 hr. No lysis occurred in trophozoites adapted with active NHS, while 78% lysis was observed in the control cultures (inactive NHS regimen). The cell cycle time of resistant amoebas was the same as that of normal ones (14 hr). Despite the large number of C-treatments (120 exposures), complete resistance to high NHS concentrations was not reached.

Resistance to C-lysis is progressively lost once C-treatment is discontinued

In order to determine whether resistance to complement killing was a permanent characteristic of adapted amoebas, we studied whether trophozoites, exposed 123 times to either NHS or inactivated NHS, changed their susceptibility to C-lysis after the

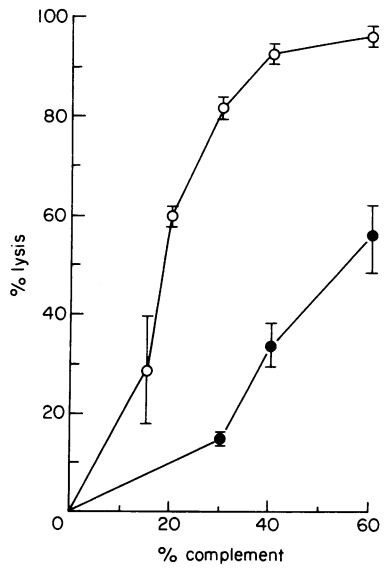

Figure 3. Dose-dependent complement lysis in *E. histolytica* HM1, clone $5C_1$ pretreated 120 times with heat-inactivated serum (○——○), and NHS (●——●), as indicated in the Materials and Methods.

Table 2. Proliferation of *E. histolytica* HM1, a clone $5C_1$, after complement lysis

Serum regimens*	Lytic assay	Viable trophozoites in subcultures ($\times 10^3$ cells)† 5 hr	29 hr
Active NHS	NHS	70±12	296±20
	(56°) NHS	67±7	272±12
Inactive NHS	NHS	19±6	96±16
	(56°) NHS	90±14	412±48

* After 4 months' regimen in 40% active or heat-inactivated NHS treatments, 76,000 and 98,000 trophozoites from respective conditions were incubated with 25% NHS in 0·5 ml at 36° for min as a lytic assay.

† Previously C-treated trophozoites were cultured for 5 hr and 29 hr to determine cell viability.

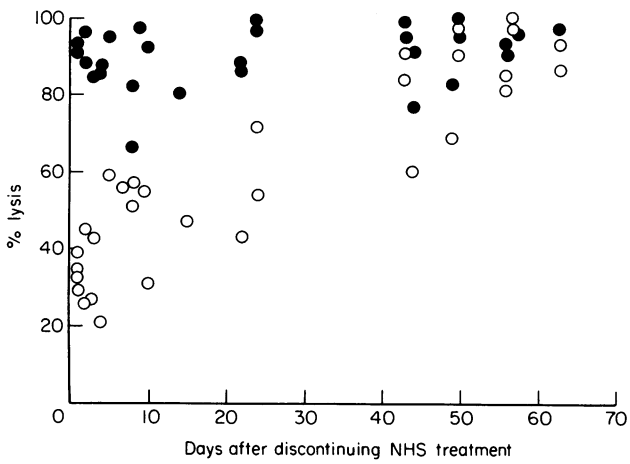

Figure 4. Reaquisition of complement sensitivity in *E. histolytica* HM1, clone $5C_1$ cultures, after discontinuing NHS treatments. Trophozoites from 123 exposures to C were subcultured without serum treatments; susceptibility to lysis was then determined in NHS (○) and inactivated NHS regimens (●) with 40% NHS at different times.

serum treatments were terminated. Complement resistance of the cultures was assessed with lytic assays over a period of 60 days following discontinuation of the serum treatments. As shown in Fig. 4, complement-adapted amoebas, grown in the absence of fresh serum, progressively regained their sensitivity to complement lysis. Forty-five to 60 days after termination of the serum treatments, the sensitivities of the adapted and control amoebas were indistinguishable. Thus, resistance to C-lysis in amoebas required continuous exposure complement and suggested that this capacity might reflect a state of cellular activation rather than a permanent mutation.

DISCUSSION

Fresh serum from non-immune individuals can kill trophozoites directly due to activation of the alternative complement pathway (Ortiz-Ortiz *et al.*, 1978; Huldt *et al.*, 1979) or classical complement pathway (Calderon & Schreiber, 1985). Hamsters, decomplemented by injection with cobra venom factor and subsequently inoculated with trophozoites, developed more severe liver abscesses than untreated animals, thus indicating that complement plays a protective role during amoeba infection (Capin *et al.*, 1980). Yet, patients with amoebiasis have normal levels of serum complement, although in some cases CH_{50} and C3 levels have been found to be slightly increased (Ganguly *et al.*, 1978) or decreased (Capin *et al.*, 1978). Thus, despite the presence of an active complement system in the host, trophozoites nevertheless remain active in producing clinical symptoms.

The results presented in this manuscript help to explain these divergent findings, since they show that some trophozoites to *E. histolytica* HM1 in axenic cultures became resistant to complement lysis after a series of treatments with NHS. Susceptibility to C remained unaltered in controls, amoebas treated with heat-inactivated NHS or left untreated. Resistance was also observed in two clones of *E. histolytica* HM1 and resistance was reversible. Taken together, the results suggest that tolerance is an acquired capacity and not a selection process of a genetically resistant subclone. The finding that the susceptibility of *E. histolytica* HK9 to complement did not decrease, despite several NHS treatments, suggests that strains with low pathogenicity are unable to develop resistance to NHS.

Although resistance to complement initially increased with every sequential NHS treatment, a limit was eventually reached such that amoebas on extended C-regimens (123 times) did not become totally refractory to high serum concentrations. With 40% NHS, about 50% of complement-adapted trophozoites were lysed, while lysis in control amoebas was over 90%. In addition to resistance in C-treated amoebas, cytotoxic and erythrophagocytic activities were increased by 50% and 100%, respectively, in *E. histolytica* HM1, yet in HK9 such functions remained unaltered (manuscript in preparation). Thus, these changes, observed after repeated serum treatments, suggest that in some strains of *E. histolytica* complement stimulates activities that facilitate its pathogenicity. Accordingly, pathogenic trophozoites in host tissues could resist complement damage by decreasing their susceptibility to lysis. Reed, Sargeaunt & Braude (1983) reported that pathogenic strains of *E. histolytica* in polyxenic cultures were resistant to human complement, while trophozoites with non-pathogenic zymodemes obtained from asymptomatic patients were susceptible to serum lysis.

Cultures of amoeba trophozoites obtained from patients represent a heterogeneous population; clones with different phagocytic, adherence and virulence activities have been isolated from a single strain of *Entamoeba* (Orozco *et al.*, 1983). Therefore, experimental culture conditions could lead to preferential cell growth of certain subpopulations, as the complement-resistant ones described in this study. However, tolerance was also induced in two different clones following serum treatment and was reversed in subcultures when NHS treatments were suspended for 7 weeks. Although it is not a stable property, its expression during 6 weeks (with a cell cycle for *E. histolytica* of 10–14 hr) rules out the possibility of a blocking effect by a heat-sensitive (56°) serum component bound to the amoebas. According to this, it seems that surviving parasites become physiologically activated by *in vitro* exposures to complement.

Future investigations are required to clarify this subject further. For example, cloning of subpopulations of a partially resistant population of amoebas may result in markedly different populations if mutation is involved, whereas persistance of heterogeneity in the organisms' response might be anticipated if an activating phenomenon is in fact responsible.

Previous reports have described other forms of activating *E. histolytica* Young *et al.* (1982) showed that membrane-active agents, such as concanavalin A, A23187 and *E. coli* LPS, induce a rapid stimulation of an ion-channel forming protein that forms aqueous pores in target membranes (Lynch, Rosenberg & Gitler, 1982). Huldt *et al.* (1979) determined that sublytic concentrations of complement increased the secretion of acid hydrolase *N*-acetyl-β-D-glucosaminidase. Therefore, this protozoan can be immediately activated and also slowly develops processes related to its ability to survive and produce tissue damage in the host. Further studies to determine if resistance to NHS is equally induced in *E. histolytica* strains of diverse virulence are necessary to define its relationship with virulence.

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