Basic Properties of *Tritrichomonas mobilensis* Hemagglutinin

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Received 21 December 1987/Accepted 26 April 1988

Tritrichomonas mobilensis is a recently described enteric protozoan of squirrel monkeys. An earlier report identified one of the metabolic products of this organism as a lectinlike hemagglutinin. Its further properties were determined in this study. Culture supernatants of *T. mobilensis* FP4190 were concentrated by ultrafiltration through a membrane with 100,000-molecular-weight cutoff. High titers of agglutinin against human erythrocytes were obtained. Incubation at 70°C for 15 min resulted in complete inactivation. Exposure to 56°C for 30 min was without effect, and only partial loss of activity was obtained during incubation for up to 18 h. Maintenance at pH 4 to 9 for 4 h at room temperature had no deleterious effect. Apparent degradation of the hemagglutinin was achieved by 18 h of contact with proteinase K, but trypsin and collagenase were essentially ineffective. Papain increased the sensitivity of the test. In the presence of this enzyme hemagglutinin was demonstrated also in cultures of *Tritrichomonas foetus* and *Tritrichomonas augusta* but not in those of *Pentatrichomonas hominis* or *Trichomonas vaginalis*.

In 1985 we reported a very high prevalence of previously undescribed intestinal trichomonads in a colony of squirrel monkeys (16). The organisms were readily detected by direct microscopic examination of fresh fecal samples and were established in cultures. Detailed study of a number of isolates resulted in description of a new species, *Tritrichomonas mobilensis* (2). Surveys of colonies maintained at geographically distant facilities also yielded a high rate of infestation (15).

Newborn squirrel monkeys were free of the parasite but acquired it at 1 to 2 months of age, either from their mothers or from cage mates (1). Sampling of a large number of adult animals failed to identify trichomonad-free individuals; these observations suggested that squirrel monkeys may be life-long carriers of *T. mobilensis*.

At present, direct effects of *T. mobilensis* on the squirrel monkey are unknown, as is the effect of metabolic products of the parasite. One such product is a lectinlike hemagglutinin demonstrated in culture fluids of *T. mobilensis* isolates (15). In this report we present some of the basic properties of the hemagglutinin. This information is deemed necessary for purification and identification of the active substance and for correlation with hemagglutinins and lectins derived from other protozoa (3, 6, 8, 9, 11-13).

MATERIALS AND METHODS

Hemagglutinin source and preparation. For determinations of basic properties the hemagglutinin was prepared from cultures of *T. mobilensis* FP4190. The organism was grown axenically in GMP medium (14) supplemented with 10% fetal bovine serum. Supernatants from 3-day-old cultures were filtered through a 0.45- μ m-pore-size membrane (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and concentrated 10-fold or more by ultrafiltration through a membrane with 100,000-molecular-weight cutoff (Amicon Corp., Danvers, Mass.). The concentration procedure was carried out at 4°C. The quality control of each preparation consisted of (i) demonstration of hemagglutinin in the crude supernatant, (ii) absence of hemagglutinin in the ultrafiltrate,

and (iii) increased hemagglutinin titer in the retentate proportionate to the extent of removal of the nonreactive ultrafiltrate. This method was consistently reliable.

Crude supernatants from 3-day-old cultures were used for hemagglutinin determinations of additional isolates of *T. mobilensis*, *Tritrichomonas foetus*, *Tritrichomonas augusta*, *Pentatrichomonas hominis*, and *Trichomonas vaginalis*. All strains were grown in GMP medium.

Diluents. For determinations of pH effect on the stability of the hemagglutinin and on the sensitivity of its assay, 50 mM citrate buffers were used for pH 2 to 6 and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffers were used for pH 7 to 11. For all other experiments the hemagglutinin was diluted in normal saline.

Enzymes. Working solutions of enzymes were prepared in normal saline. All were purchased from Sigma Chemical Co., St. Louis, Mo., and their respective product numbers were as follows: proteinase K, P-0390; trypsin, T-0646; collagenase, C-0130; papain, P-3375.

Erythrocytes. Human type O erythrocytes from healthy donors were prepared in normal saline and used as previously described (15).

Hemagglutinin assay. Hemagglutination tests were performed as described previously (15), with the following modifications: for routine purposes, normal saline was used as diluent in place of phosphate-buffered saline (pH 7.2), the test results were scored after 1 h instead of overnight incubation at room temperature, and for demonstration of the augmenting effect of papain 1.25 mg of the enzyme per ml was added to the diluent.

Inactivation experiments (enzymes, pH, heat) were repeated three times, and individual results were in agreement within one twofold dilution. Other data were similarly consistent in repetitive runs.

RESULTS

Effect of enzymes on the hemagglutinin. Graded concentrations of enzymes were added in equal volumes to sets of serial dilutions of concentrated hemagglutinin with a predetermined titer of 1:1,024. Erythrocytes were added after incubation at 37°C for 18 h.

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TABLE 1. Effect of enzymes on activity of concentrated hemagglutinin^a

Enzyme and concn (mg/ml)	Agglutination in the following dilutions of concentrate:								Saline control
	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	
Proteinase K									
0.025	+	+	+	+	0	0	0	0	0
0.125	+	+	+	+	0	0	0	0	0
0.250	+	±	0	0	0	0	0	0	0
0.500	+	0	0	0	0	0	0	0	0
1.000	+	0	0	0	0	0	0	0	0
Trypsin									
12.5	+	+	+	+	0	0	0	0	0
25.0	+	+	+	0	0	0	0	0	0
Collagenase									
1.25	+	+	+	+	+	0	0	0	0
5.0	+	+	+	+	0	0	0	0	0
Papain									
1.25	+	+	+	+	+	+	+	+	0
2.5	+	+	+	+	+	+	+	+	0
Control (normal saline)	+	+	+	+	+	0	0	0	0

^a Sets of agglutinin serially diluted in saline were treated with enzymes in final concentrations as shown. Erythrocytes were added after 18 h of incubation at 37°C. Marked reduction of activity was achieved with proteinase K, trypsin and collagenase had a minimal effect, and a significant increase in agglutinin was obtained with papain.

Table 1 shows a dose-dependent loss in hemagglutinin titer after exposure to proteinase K. A lesser decrease of activity was also associated with trypsin and collagenase. In contrast, addition of papain led to an eightfold increase.

Enhancement of hemagglutination by papain. Optimal conditions for increase of hemagglutinin titer in the presence of papain were determined as follows. Papain in uniform final concentration (2.5 mg/ml) was added to five replicate sets of hemagglutinin dilutions. In sets 1 and 2 the enzyme was introduced at 3 and 1 h before addition of erythrocytes, respectively; in set 3 the enzyme was added just (<5 min) before erythrocytes; in the remaining two sets the order of addition of papain and erythrocytes was reversed, i.e., erythrocytes were added first and the enzyme was introduced at 30 min (set 4) and 2 h (set 5) thereafter; no papain was added to the control (set 6).

In the control set the titer was 1:512. In all instances where papain was added this value increased to 1:4,096. The results were not influenced by the length of time the enzyme interacted with the hemagglutinin before erythrocytes were added. Even when the order of the addition of papain and erythrocytes was reversed (sets 4 and 5) the same eightfold rise in titer was obtained.

The enhancement of hemagglutination by papain was dose dependent. This was demonstrated by adding graded amounts of papain to matched sets of the hemagglutinin serially diluted beyond the predetermined extinction point. The enzyme was added just before the addition of erythrocytes.

The lowest concentration of papain causing a twofold rise in titer was 0.31 mg/ml (Fig. 1). Further increases of test sensitivity were associated with successively higher amounts of papain. The limit of titer increase was reached with 1.25 mg/ml. The effect was not due to a direct interaction between the enzyme and the hemagglutinin. More likely, papain altered or activated the receptors on the erythrocytes. This is suggested in the last column; washed erythrocytes were maintained for 1 h in saline containing 2.5

mg of papain per ml; after three washes in 10 volumes of saline the same increased titer was obtained with these erythrocytes as with normal erythrocytes in the presence of papain.

Effect of pH on the hemagglutinin. Concentrated stock hemagglutinin was diluted 1:10 in saline (control) and in buffers of pH 2.0 to 11.0, in one-unit increments. After 4 h of incubation at room temperature the pH of samples was adjusted to neutral with 0.1 M NaOH or HCl. Further twofold dilutions, ranging from 1:20 to 1:5,120, were made both in normal saline and in the original buffers to which the samples were exposed.

Titration in saline (Fig. 2A) showed that the hemaggluti-

FIG. 1. Dose-response enhancement of hemagglutination by papain. Increasing amounts of papain were added to hemagglutinin diluted in saline. Normal erythrocytes were added immediately thereafter. In the right-hand column, erythrocytes were first treated with papain and then washed and added in saline suspension.

FIG. 2. Effect of pH on hemagglutinin. Samples of hemagglutinin were diluted 1:10 in buffers of pH 2.0 to 11.0 and in saline (control). After 4 h of incubation at room temperature the pH of the samples was adjusted to neutrality. Further dilutions were made in saline (A) and in the same buffers to which the samples were exposed (B). U, Unsatisfactory.

nin remained stable over the pH range from 4.0 to 9.0. Partial decrease was noted at pH 3.0 and 10.0. No activity was detected after exposures to pH 2.0 or 11.0. The assay appeared to be more sensitive at an acid pH (Fig. 2B). The highest sensitivity was at pH 5.0. Endpoints obtained at pH 4.0 and 6.0 and in the saline control were one twofold dilution lower. A further twofold decrease occurred at pH 7.0, 8.0, and 9.0. At pH 2, 3, 10, and 11 lysis of erythrocytes precluded this assay.

Thermal inactivation of the hemagglutinin. Thermostability of the hemagglutinin was evident by preservation of titer in culture supernatants frozen at -20°C for 18 months; 6 weeks of continuous incubation at 37°C was required to obtain a fourfold titer decrease (data not shown). Inactivation studies at higher temperatures were as follows.

Portions of concentrated hemagglutinin (titer, 1:4,096) were heated at 56 and 70°C for intervals ranging from 5 min to 18 h and assayed in saline containing 1.25 mg of papain per ml. At 56°C there was no loss of activity during the first 30 min of incubation (Fig. 3). A twofold decrease was present at 1 and 2 h. At 18 h the titer was 1:512.

In contrast, there was a precipitous loss of activity after incubation at 70°C ; i.e., the first (5-min) sample showed an eightfold loss, with the same trend at 10 and 15 min. No activity was detected in samples tested at 30 min and subsequent intervals.

Specificity of the hemagglutinin. The augmenting effect of papain (Fig. 1) was utilized for the detection of hemagglutinin in unconcentrated culture supernatants of additional strains of *T. mobilensis* and of other trichomonads.

In saline without papain, minimal to moderate titers were obtained with tritrichomonads but not with *P. hominis* or *T.*

FIG. 3. Thermal inactivation of hemagglutinin. Portions of hemagglutinin stock were incubated for 5 min to 18 h at 56 and 70°C . Each sample was diluted in saline and assayed in the presence of papain.

vaginalis (Table 2). In the presence of papain the titers of all tritrichomonads were markedly elevated, whereas members of the other two genera remained negative.

DISCUSSION

T. mobilensis has been described only recently (2), and knowledge of its characteristics is still sparse. One of its properties described earlier (15) is the production of a lectinlike hemagglutinin. This finding was of special interest, since among protozoa only *Giardia lamblia* (3, 8, 9) and *Entamoeba histolytica* (6, 11–13) are known to produce lectins.

TABLE 2. Specificity of the hemagglutinin^a

Organism and strain	Endpoint titer	
	Without papain	With papain
<i>T. mobilensis</i>		
ATCC 50116 ^b	1:1	1:64
SM776 ^c	1:2	1:64
SM227 ^c	1:1	1:32
SM4295 ^c	1:32	1:512
<i>T. foetus</i>		
ATCC 30003 ^b	1:1	1:32
<i>T. augusta</i>		
ATCC 30077 ^b	1:4	1:128
<i>P. hominis</i>		
ATCC 30000 ^b	0	0
CoRhe B-144 ^c	0	0
Dg4790 ^c	0	0
<i>T. vaginalis</i>		
Baltimore 42 ^d	0	0
V3402 ^c	0	0
Medium control	0	0

^a Supernatants from 3-day-old cultures of tritrichomonads, pentatrichomonads and *T. vaginalis* were tested for the presence of agglutinin in saline only and with papain (1.25 mg/ml). All tritrichomonads tested positive, and the titers were markedly increased in the presence of papain.

^b Obtained from American Type Culture Collection.

^c Original isolates from our laboratories.

^d Received through the courtesy of B. M. Honigberg.

The inhibitory effect of a specific sugar is generally required for characterization of a hemagglutinin as a lectin. In a previous study (15) none of 10 arbitrarily chosen carbohydrates had such effect. Clearly, determinations of additional properties of the hemagglutinin were needed to efficiently proceed toward a more precise definition of its characteristics and identity.

The hemagglutinin molecule is likely to contain some protein, as indicated by loss of activity after exposure to proteinase K (Table 1). Trypsin and collagenase had little direct effect on the hemagglutinin assay. Papain significantly increased the sensitivity of the test, perhaps by its action upon erythrocytes (Fig. 1). Similar uses of papain (4, 7, 17) or trypsin (3, 5, 8, 10) treatment have been reported for assay of other agglutinins and lectins.

The hemagglutinin is remarkably stable at moderate temperatures. Similarly, changes of pH over a rather wide range had no deleterious effect. These properties suggest that several procedures can be used for purification and more definitive characterization of the hemagglutinin. They also indicate that the *T. mobilensis* hemagglutinin differs substantially from that of recognized lectins of protozoan origin. Thus, *E. histolytica* lectin was very unstable at 37°C, and even at 4°C considerable loss of activity was observed (6, 11). *G. lamblia* lectin required enzymatic activation (8).

With introduction of papain into the assay system all isolates of *T. mobilensis* tested thus far produced a clearly demonstrable hemagglutinin. Similar results were also obtained with *T. foetus* and *T. augusta* but not with two other genera of trichomonads. This finding suggests that production of hemagglutinin may be a common property of organisms belonging to the genus *Tritrichomonas*, and its demonstration may be of use in discriminating between trichomonads. Immunohistochemical and other procedures can be expected to reveal differences in the hemagglutinins of the three species of tritrichomonads and, possibly, strain differences within the individual species. Such information may contribute to a better understanding of the pathogenicity of these organisms.

ACKNOWLEDGMENTS

We express our gratitude to Bronislaw M. Honigberg for kindly supplying the *T. vaginalis* Baltimore 42 strain and to Joe Ann Thomas for preparation of the manuscript.

This study was supported by Public Health Service grant RR 01254 from the National Institutes of Health and by the South Alabama Medical Science Foundation.

LITERATURE CITED

1. Brady, A. G., F. F. Pindak, C. R. Abee, and W. A. Gardner. 1988. Enteric trichomonads in squirrel monkeys (*Saimiri sciureus*).

2. Culbertson, D. E., F. F. Pindak, W. A. Gardner, and B. M. Honigberg. 1986. *Tritrichomonas mobilensis*, n. sp. (Zoomastigophorea: Trichomonadida) from the Bolivian squirrel monkey *Saimiri boliviensis boliviensis*. J. Protozool. 33:301-304.
3. Farthing, M. J. G., M. E. A. Pereira, and G. T. Keusch. 1986. Description and characterization of a surface lectin from *Giardia lamblia*. Infect. Immun. 51:661-667.
4. Gilboa-Garber, N. 1972. Inhibition of broad spectrum hemagglutinin from *Pseudomonas aeruginosa* by *D*-galactose and its derivatives. FEBS Lett. 20:242-244.
5. Gordon, J. A., N. Sharon, and H. Lis. 1972. Binding of soybean agglutinin by normal and trypsin-treated red blood cells. Biochim. Biophys. Acta 264:387-391.
6. Kobilier, D., and D. Mirelman. 1980. Lectin activity in *Entamoeba histolytica* trophozoites. Infect. Immun. 29:211-225.
7. Kuhns, W. J., and A. Bailey. 1950. Use of red cells modified by papain for detection of Rh antibodies. Am J. Clin. Pathol. 20:1067-1069.
8. Lev, B., H. Ward, G. T. Keusch, and M. E. A. Pereira. 1986. Lectin activation in *Giardia lamblia* by host protease: a novel host-parasite interaction. Science 232:71-73.
9. Lev, B., H. Ward, and M. E. A. Pereira. 1986. Prolectin activation in *Giardia lamblia*, p. 301-317. In D. Mirelman (ed.), Microbial lectins and agglutinins: properties and biological activity. John Wiley & Sons, Inc., New York.
10. Lis, H., and N. Sharon. 1973. Soy bean (*Glycine max*) agglutinin. Methods Enzymol. 28(partB):360-365.
11. Lushbaugh, W. B., A. B. Kairalla, J. R. Catney, A. F. Hofbauer, and F. E. Pittman. 1979. Isolation of a cytotoxin-enterotoxin from *Entamoeba histolytica*. J. Infect. Dis. 139:9-17.
12. Mattern, C. F. T., D. B. Keister, and P. C. Natovitz. 1980. *Entamoeba histolytica* "toxin": fetuin neutralizable and lectin-like. Am. J. Trop. Med. Hyg. 29:26-30.
13. Mirelman, D., and J. I. Ravdin. 1986. Lectins in *Entamoeba histolytica*, p. 319-334. In D. Mirelman (ed.), Microbial lectins and agglutinins: properties and biological activity. John Wiley & Sons, Inc., New York.
14. Pindak, F. F., W. A. Gardner, Jr., and M. Mora de Pindak. 1986. Growth and cytopathogenicity of *Trichomonas vaginalis* in tissue cultures. J. Clin. Microbiol. 23:672-678.
15. Pindak, F. F., W. A. Gardner, Jr., M. Mora de Pindak, and C. R. Abee. 1987. Detection of hemagglutinins in cultures of squirrel monkey intestinal trichomonads. J. Clin. Microbiol. 23:609-614.
16. Pindak, F. F., M. Mora de Pindak, C. R. Abee, and W. A. Gardner, Jr. 1985. Detection and cultivation of intestinal trichomonads of squirrel monkeys (*Saimiri sciureus*). Am. J. Primatol. 9:197-205.
17. Smith, D. F., G. Neri, and E. F. Walborg, Jr. 1973. Isolation and partial chemical characterization of cell-surface glycopeptides from AS-30D rat hepatoma which possess binding sites for wheat germ agglutinin and concanavalin A. Biochemistry 12:2111-2118.