Complement Activating Factor(s) of Trypanosoma lewisi: Some Physiochemical Characteristics of the Active Components

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ABSTRACT

Of the complement activating factors present in Trypanosoma lewisi, the major component, a carbohydrate containing substance was further investigated. This component was found to have a lag time of complete activation of 2 CH_{50} units of bovine complement of approximately 15 minutes while 1% trypsin (a known activator of complement, used as a control system) was capable of instant consumption of a similar quantity of complement. In addition, the complement activating factor of trypanosomes was observed to be stable at 100°C for 15 minutes and over a pH range of 3.0 to 11.0. Thin layer chromatography studies suggested that at least part of the active component contained lipid, perhaps indicating that it may be glycolipid in nature.

RÉSUMÉ

Cette expérience visait à étudier plus à fond une substance contenant un hydrate de carbone et représentant le principal constituant des facteurs qui activent le complément et qui sont présents chez Trypanosoma lewisi. On réalisa que cette substance prenait environ 15 minutes pour activer complètement deux unités CH_{50} de complément bovin, tandis que de la trypsine 1%, une substance reconnue pour activer le complément et utilisée comme témoin, pouvait consommer instantanément une quantité comparable de complément. De plus, le facteur des trypanosomes qui active le complément s'avéra stable à 100°C pendant 15 minutes, ainsi qu'à un pH qui variait de 3.0 à 11.0. La chromatographie permit de penser qu'au moins une fraction du composé actif contenait des lipides, indice probable de sa nature glycolipidique.

INTRODUCTION

In previous communications, complement activating factors of trypanosomes were described (9, 10). These factors, one of which was thought to be a protein, perhaps an enzyme while the major component was shown to be a carbohydrate containing material, are released from the trypanosomes upon disruption of the cell. While polysaccharide materials which are capable of activating complement are not unusual, most have been shown to activate the alternate pathway (for example, streptococcal polysaccharides have been shown by Fine (3), Winkelstein *et al* (14), Reed *et al* (11) and Tauber *et al* (12, 13) to activate the bypass mechanism of complement consumption). Unlike these factors and parallel to those described by Berrens *et al* (2) obtained from house dust, the trypanosomal complement activator consumed the first component (C1) of bovine complement (9).

This communication deals with further

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attempts to support the initial characterization of this trypanosomal factor as being a carbohydrate containing a lipid moiety but very little, if any, protein.

MATERIALS AND METHODS

Trypanosomes

Trypanosoma lewisi were harvested and purified from the blood of infected rats, according to the method of Lanham and Godfrey (7). The organisms were suspended in 0.04 M phosphate buffer pH 8.0 containing 0.04 M NaCl and 1% glucose (PSG) to a concentration of 1 x 10^9 organisms per ml. The suspended organisms were frozen and thawed three times and disrupted using a Virtis homogenizer. The suspension was then centrifuged at 11,000xg for ten min yielding a supernatant solution and a pellet which was resuspended in PSG to the original volume of the suspension. These preparations were stored at 4°C until needed.

Trypsin

Trypsin (Fisher Scientific Co.) was made up to 1% (W/V) in 0.15 M NaCl pH 7.0.

Titration of Complement

Hemolytic titrations for complement were performed according to the method of Barta and Barta (1). Rabbit erythrocytes were washed three times and made up to 5% in veronal buffer pH 7.3 containing 0.15 M NaCl 0.003 M sodium bicarbonate, 0.01 M EDTA (disodium salt) and 0.1% gelatin (VBG-EDTA). Sheep hemolysin was diluted optimally in veronal buffer pH 7.3 containing 0.15 M NaCl, 0.003 M sodium bicarbonate, 5 mM Mg^{++} and 1.2 mM Ca^{++} (VB). Equal amounts of the 5% erythrocytes and 1:65 hemolysin were allowed to react at room temperature for 45 min. The sensitized cells were then washed once with VBG-EDTA, twice with VB and resuspended to 5% with VB.

Whole bovine serum diluted with VB was used as the complement source so that 0.4 ml of 1:8 would contain 2 CH_{50} units of hemolytic complement activity.

Titration of Complement Activating Factors of Trypanosomes

Volumes of 0.4 ml of sensitized erythrocytes were added to 1.2 ml amounts of VB in 50 x 5 mm glass tubes. To these tubes 0.4 ml volumes of complement incubated with the trypanosomal factors or with an equivalent volume of saline were added. Trypanosome factors or saline had been previously incubated with complement at 37°C for 30 min. Fresh complement was kept in an ice bath and added directly. A control containing an appropriate amount of 0.15 M NaCl (equal to the amount of sample used) in addition to 0.4 ml of complement indicated the maximum amount of lysis (100%) possible in each test. Tubes containing erythrocytes, buffer and complement were then incubated at 37°C for one hour after which the amount of hemoglobin present in the supernatant was determined in a Unicam SP1800 spectrophotometer at 541 nm. Controls were included to compensate for readings due to complement colour and noncomplement mediated lysis.

Generation of Active Component

Generation of the complement activating component(s) present in the *T. lewisi* pellet and supernate and in trypsin was followed over a 30 min time period. Tubes containing 0.1 ml amounts of sample and 0.4 ml of complement were analyzed every five min. Controls containing 0.1 ml of 0.15 M NaCl and 0.4 ml of complement were included. After addition of the complement the first set of tubes for zero minute time were immediately placed in an ice bath to await titration. Remaining tubes were incubated at 37°C and a sample set removed every five minutes for titration by photometric determination of the amount of hemoglobin released from the sensitized erythrocytes. In the case of the *T. lewisi* pellet and trypsin samples, centrifugation at 5,000 xg for three min was necessary to remove the particulate matter before titrating the 0.4 ml of the reacted complement mixture. Lysis of each sample was calculated in rela-

tion to the saline-complement control included for that particular time interval.

Effect of pH on Active Component

The effect of pH on the complement activating component(s) present in the *T. lewisi* pellet, supernate, trypsin and a H_2O control was examined over a pH range of 0.5 to 11.0. Volumes of 0.5 ml of each sample were treated with 0.1 M NaOH or 0.1 M HCl to include pH 0.5, 3, 5, 7, 9 and 11. After adjustment to their appropriate pH, the samples were allowed to stand at room temperature for 30 min. The pellet samples were washed once with 0.15 M NaCl before adding 0.4 ml of complement. The supernate, trypsin and H_2O samples were dialyzed at 20°C against deionized water for two and one-half hours after which 0.2 ml was removed to react with 0.4 ml of complement. After reaction with the complement the samples were titrated. Amounts of lysis were determined as described in the Materials and Methods section in comparison to a control containing 0.1 ml of 0.15 M NaCl and 0.4 ml of complement.

Effect of Temperature on Active Component

In addition to samples of trypsin, trypanosome pellet and supernate an additional sample containing *T. lewisi* supernate treated with 10% (final concentration, W/V) trichloroacetic acid (TCA) for 15 min at 20°C was included. The precipitate resulting from this treatment was removed by centrifugation at 11,000 xg for ten min, after which the supernates were dialyzed against 2 *l* of distilled water at 4°C. Volumes of 0.2 ml of each sample were incubated for 15 min at each of 36°C, 56°C and 100°C. To each sample was added 0.4 ml of complement, after which the reacted complement was titrated. A 100% lysis control containing 0.2 ml of saline and 0.4 ml of complement was included in parallel to each sample to indicate maximum lysis under these conditions.

Estimation of Carbohydrate and Protein Contents

Carbohydrate (hexose) levels were estimated by the Anthrone technique as outlined by Kabat and Mayer (5). Standard curves ranging from 250 μg to 15.75 μg of dextrose and of sucrose were used for comparison.

Protein contents of the supernate was measured by ultraviolet spectrophotometry using wave lengths of 280 and 260 nm (Unicam SP 1800 spectrophotometer).

Thin Layer Chromatography

The supernate from the trypanosome preparation suspended in water was extracted with chloroform:methanol (2:1 v/v)

TABLE I. Time Course of Prevention of Lysis by Complement Activation by Trypanosome Cellular Debris (pellet), Trypanosome Supernate and Trypsin

	% Lysis						
	Time (min)						
	0	5	10	15	20	25	30
Pellet	54.2	22.3	16.1	0	0	0	0
Supernate	76.2	15.9	4.7	3.2	2.6	2.9	2.4
Trypsin (1%)	0	0	3.0	2.0	1.9	2.4	2.5

TABLE II. The Effect of pH on the Ability of Trypanosome Supernate, Cellular Debris (pellet) and Trypsin on their Ability to Prevent Lysis by the Activation of Complement

	% Lysis					
	pH					
	0.5	3.0	5.0	7.0	9.0	11.0
Pellet	17.6	8.9	6.2	4.9	2.5	2.5
Supernate	14.3	12.9	0	0	5.8	7.7
Trypsin 1%	79.6	4.2	6.7	8.7	7.5	5.5
H_2O (control)	100	96.0	100	100	96.0	93.3

TABLE III. The Effect of Temperature on the Ability of Trypanosome Supernate, Cellular Debris (pellet) and Trypsin on their Ability to Prevent Lysis by the Activation of Complement

Sample	Treatment	% Lysis		
		Temperature		
		37°C	56°C	100°C
Pellet	H_2O	0.13	0.13	28.3
Supernate	H_2O	4.7	4.7	5.4
Supernate	TCA (10%)	3.4	3.4	2.9
Trypsin (1%)	—	15.6	37.0	100
H_2O (control)	—	100	100	100

for 15 hrs at 56°C using ten volumes of solvent per volume of supernate. The aqueous and solvent phases were separated by centrifugation at 1000 xg for ten min at 4°C. The separated solvent phase was evaporated to dryness and resuspended in a mixture of chloroform:methanol:water (65:24:4 v/v) which was then chromatographed in the same solvent system on a glass plate coated with silicagel G (0.25 mm thickness). Upon completion, the chromatogram was dried and developed in a chamber saturated with iodine vapour. The Rf values of any apparent spots were determined and the spots were marked. After the iodine had faded from the spots they were scraped off and the materials were extracted in chloroform. The silicagel was removed by centrifugation at 1000 xg for 10 min at 4°C and the chloroform phase was again evaporated to dryness. The materials were resuspended in saline and their ability to activate complement was tested by the method described in the Materials and Methods section.

RESULTS

The complement activating factor(s) present in the cellular debris pellet and in the supernate of disrupted *Trypanosoma lewisi*, while capable of partly activating complement at time zero, are not fully so until between ten and 15 min incubation with complement. For comparison, 1% (W/V) of trypsin can activate the complement cascade immediately. These findings are summarized in Table I.

From the data presented in Table II, it is clear that while the active component(s) are somewhat unstable under extreme acidic conditions (pH 0.5) and less so at a pH of 3.0, they are generally stable at slightly acid conditions and even a highly alkaline pH has little effect. Trypsin on the other hand is highly labile at a pH of 0.5 but stable over a pH range of 3.0 to 11.0. Buffer of the same pH but containing no trypanosomes but treated in a similar fashion to the test materials had very little effect on the efficacy of the bovine complement system. From the data presented in Table III it is clear that the complement activating factors present in the cellular debris pellet prepared from disrupted *Trypanosoma lewisi* is partly heat labile at 100°C but not at 56°C. The supernate of the same trypanosome preparation, whether treated with water or 10% TCA (final concentration W/V), is not affected by boiling for 15 min. Trypsin (1% W/V) which was included for comparison was partly labile

TABLE IV. Activation of Complement by Trypanosome Supernate Extracted with Chloroform:Methanol and Chromatographed on a Thin Layer of Silicagel G. Rf Values Indicate the Location of Each Spot on the Plate in Relation to the Solvent Front which Moved 121 mm

Sample	Treatment	Rf value	% lysis
Supernate	—	—	0
Supernate	Chloroform: methanol extraction-solvent phase	—	0
Supernate	Aqueous phase	—	0
Supernate	Chloroform: methanol extract chromatographed on thin layer plate	0.057	0
		0.087	12.5
		0.107	53.4
		0.223	83.9
		0.264	87.5
		0.388	100.0
		0.661	100.0
		0.769	100.0
		0.884	100.0

at 56°C and was completely inactivated in terms of its ability to activate the complement cascade at 100°C.

Carbohydrate (hexose) and protein determinations gave the following results: the trypanosomal supernatant and pellet contained 9.8 and 9.6 μg carbohydrate (dextrose equivalents) per ml respectively and no measurable protein. The trypsin suspension contained no measurable carbohydrate and while the optical density could not be measured due to the particulate nature of the trypsin its protein content by weight was 10 mg/ml.

Thin layer chromatography of the supernate gave the results presented in Table IV. It is clear from this data that only part of the supernate was soluble in chloroform: methanol and that a proportion of the active material remained in the original aqueous phase. The active material which was soluble in chloroform:methanol did not move or only slightly so in the chloroform: methanol:water, suggesting that the material perhaps is a large molecule or absorb to the matrix.

DISCUSSION

Previous evidence has been produced to indicate that the complement activating factor(s) of trypanosomes may be a carbohydrate containing material (10). Evidence reported in this communication supports the initial findings and it would appear that a second component, perhaps an enzyme, which is capable of activating complement may also be present in trypanosome cellular debris. Thus heating to 100°C of the trypanosome pellet reduced the ability of the active material to prevent lysis by 28% (Table III), while such treatment had no effect on the ability of the supernate to reduce lysis. On the other hand, since no enzyme activity has been demonstrated in the pellet material it may equally well be true that the loss of activity is due to the active component being bound to protein and thereby destroyed by heating. Similar treatment of trypsin completely abolished its ability to activate bovine complement.

While treatment of both the trypanosome pellet and supernate under highly acidic conditions had some detrimental effects on the active substances, such effects were minimal when compared to the destruction of trypsin by acid (Table II). The complement activating capacity of the trypanosome materials and of the trypsin was not reduced by alkaline pH values up to 11.0.

In comparison to 1% trypsin, trypanosome pellet and supernate were relatively slow to completely activate $2CH_{50}$ units of bovine complement (Table I). Thus both fractions required ten to 15 min of incubation for complete activation, while trypsin did so immediately. This difference may be the result of at least two factors: The supernate and the pellet materials contained approximately 10 μg/ml of carbohydrate material and no measurable protein, while the trypsin suspension was 1000 times more concentrated. The time difference may therefore be related to the concentration of active material. Secondly, while the trypanosome material has been shown to activate the bovine complement cascade at the C1 (first component) level (9), trypsin is generally thought to activate the alternate pathway at the C3 level (4).

As is evident in Table IV, extraction of the supernate with chloroform:methanol resulted in an only partial extraction of the complement activating material. This would indicate that the factor is partly lipid and partly carbohydrate perhaps in the form of a glycolipid.

While it is relatively unusual for carbohydrate containing substances to activate the complement cascade by the first component, such has been reported to be the case with house dust (2). Thus the carbohydrate-lysine material from house dust is not unlike the factor produced by trypanosomes in their ability to activate C1, except that house dust carbohydrate was unable to activate guinea pig C1, whereas the trypanosome factor was fully able to do so in the case of *T. lewisi* (9). However, bacterial lipopolysaccharide, in itself a potent activator of the complement cascade, has been shown to contain a polysaccharide capable of activating C3 and lipid A, which can activate C1 (8). Such may be the case *in vivo* as well in that cattle infected with *T. congolense* have markedly decreased C1 and C3 levels in their sera (Unpublished observations). Part of the decrease of these complement component levels may be a result of immune complex formation. However, part may be caused by the trypanosome itself or materials from lysed parasites.

Total hemolytic complement levels in trypanosome infected cattle has been shown

to be decreased (6), thereby perhaps rendering the animal unable to eliminate trypanosomes effectively. Also lower levels of hemolytic complement may be partly responible for the frequent occurrence of secondary infections in trypanosome infected animals. However, the overall effect of the complement activating factors possessed by trypanosomes on the pathogenesis of the disease remains unexplored.

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