Hodam et al.[23] have reported that another lipophilic extracellular signal molecule prostaglandin E_2, binds to uterine membranes. Our studies on binding kinetics are consistent with these observations. The nature and origin of these binding components and the manner in which they may contribute to the recognition and mediation of entry of DHA-S into the cell remain to be determined.

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Inhibition of IgM antibody-mediated aggregation of *Trypanosoma gambiense* in the presence of complement

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Abstract. This paper deals with the immune reaction between *Trypanosoma gambiense* and monoclonal IgM mouse antibody at equivalence with or without rabbit complement. Antibody-mediated trypanosome clumps formed in the absence of complement, and were readily dissociated by complement to become free. In the presence of complement, on the other hand, *T. gambiense* were not aggregated by the antibody. Free parasites adhered readily to cultured peritoneal macrophages. Complement-mediated dissociation of the clumped trypanosomes in the equivalence area released a large number of previously bound surface antigens. These antigens were capable of binding again to fresh IgM antibody. Experimental results further indicated that the complement system caused a functional alteration, changing the multivalent nature of the IgM antibody in the immune complex into a univalent one. This phenomenon is of great advantage to the infected host in clearing pathogens in vivo, as it allows more antibodies to attach to trypanosomes and subsequently initiate complement activity.
Key words. Trypanosoma gambiense; monoclonal IgM antibody; immune reaction; dissociation; immunophagocytosis.

Animals challenged with crude homogenates of blood trypanosomes were protected against the homologous strain of trypanosomes[1]. Antiserum passively transferred from immunized donors was also effective in protecting recipients against trypanosomes. These in vivo trials were initially suggested by in vitro observations on the immune reaction between the trypanosome, the antiserum and complement. In vitro, *T. gambiense* cells were aggregated by heat-inactivated antiserum[1]. The clumped trypanosome masses formed by heat-inactivated antiserum were dissociated by complement[2]. While the mechanism of the dissociation was obscure, it was reported recently that the dissociation of aggregates mediated by rabbit IgG antibody[3] or mouse mAb IgG 3 antibody[4] was associated with a functional change in the nature of the bivalent antibodies in the immune complex, which changed into univalent ones in the presence of complement.

There are several classes of immunoglobulins. Both IgG and IgM antibodies to *T. gambiense* were conspicuously effective in clearing trypanosomes from infected mice, though the latter is known to be more effective than the former for elimination of pathogens [5]. This is associated with the fact that the IgM antibody is different from the IgG antibody in characteristics such as molecular weight and the number of antigen-binding sites [6].

This paper enquires further into the mechanism by which host immune protection mediated by a monoclonal IgM antibody to a trypanosome surface antigen is facilitated. It suggests that in the aggregation reaction, the primary bonds between trypanosome surface antigen and monoclonal IgM antibody become affected by complement, and that the activated complement covalently binding to the IgM antibodies renders the multivalent IgM antibody functionally univalent. The advantages of such a phenomenon in helping the host to clear the trypanosomes are discussed.

Materials and methods

Trypanosomes. A cloned population of *Trypanosoma gambiense*, Wellcome strain, Type O, was used. The trypanosomes were maintained in 18–20-g mice (dd strain) by serial transfer at 3-day intervals.

Collection and separation of trypanosomes. Wistar male rats (250 g) were infected intraperitoneally (i.p.) with 5×10^7 *T. gambiense* from an infected mouse. The blood was collected at peak parasitemia by heart puncture. Trypanosomes free of host blood cell components were obtained by means of a DEAE-cellulose column [7]. The trypanosomes were washed 5 times with 1% glucose phosphate-buffered saline (GPB), ionic strength 0.271, pH 7.5, and centrifuged at 800 × g for 10 min. After final centrifugation, the parasites were resuspended in a suitable volume of ice-cold GPB. Parasites were counted in a hemocytometer.

Cells and culture medium. Myeloma cells (P3-X63Ag8-653) were purchased from Dainihon-Seiyaku Ltd and maintained in GIT culture medium (Nihon Seiyaku Ltd).

Immunization and hybridization. Six-week-old female BALB/c mice were inoculated i.p. with 1×10^4 living *T. gambiense* in 0.2 ml GPB. 72 h later, the infected mice were given 0.5 ml of fresh normal human serum i.p. to completely cure the trypanosome infection. 72 h after the treatment, splenic lymphocytes were fused with myeloma cells at a ratio of 5/1 in polyethylene glycol 1540 (Katayama Kagaku Ltd). Selections of hybridoma were done using GIT culture medium containing 1×10^{-7} M aminopterin (SIGMA). Screening for the production of antibodies to *T. gambiense* was done by an aggregation reaction as described below. The clone, designated YMMH, was determined to produce IgM antibodies by means of Ouchterlony plate examination and 2-mercaptoethanol (2 ME) treatment as described below. For the plate examination, antiserum to mouse IgM molecules was purchased from Cappel Laboratories. This clone was subcloned twice by limiting dilutions and expanded in tissue culture.

Aggregation test. Two-fold serial dilutions of YMMH supernatant were prepared, and 0.5 ml then mixed with an equal volume of GPB containing 1×10^8 *T. gambiense*. After 10 min at 23 °C, the agglutination titer was determined under the microscope (× 200) as the highest dilution of supernatant in which agglutination occurred. The agglutination titer of the supernatant was 1:128.

Determination of the region of equivalence (optimal antigen-antibody ratio). Volumes of 0.2 ml of parasite suspensions at different concentrations were mixed with 0.1 ml of YMMH supernatant. Ten min later, the region of equivalence was determined as the highest dilution of parasite suspension in which all trypanosomes had clumped. The suspensions containing more trypanosomes than the equivalent suspension were considered as containing antigen excess. The suspensions containing less trypanosomes than the equivalent suspension were considered as containing antibody excess.

Presensitization of trypanosomes. Trypanosome suspensions were mixed with supernatant of YMMH in the region of extreme antibody excess where agglutination of trypanosomes did not occur (experiment no. 9, table 1). Ten min later, the sensitized trypanosomes were washed three times with ice-cold GPB by centrifugation at 800 × g for 5 min. After the final centrifugation, the trypanosomes were resuspended in ice-cold GPB and divided into two groups of suspensions (table 3). One suspension was mixed with fresh normal rabbit serum as the source of complement. After 5 min, the suspension was mixed with 10 units of cobra venom factor (CVF) to inactivate excess complement. This mixture was designated as IgM-complement-binding trypanosome suspension. The other suspension, as a control, was mixed with saline alone instead of fresh normal rabbit serum. Five min later, it was treated with CVF. This was designated as IgM-binding trypanosome suspension. Both groups of trypanosomes were immediately used for the experiment.

Complement depletion of fresh normal rabbit serum. Fresh rabbit serum deficient in C 3 was prepared by incubation of fresh normal rabbit serum with 10 units of cobra venom factor (CVF) from *Naja naja*, which was purified by DEAE-cellulose column chromatography and Sephadex G-200 gel filtration [8].

Assay of haemolytic activity of CVF-treated normal rabbit serum. To assess the haemolytic capacity of the CVF-treated normal rabbit serum used, 6×10^6 sheep erythrocytes, optimally sensitized with rabbit haemolysin and suspended in 0.25 ml Mg^{++} gelatin veronal buffer [9], were added to 0.75 ml of the reaction mixtures, which contained the same cations, inhibitors, and serum constituents used in the experiments.

Reduction and alkylation of IgM antibody. The globulins in YMMH supernatant were reduced in 2 ME followed by alkylation with iodoacetamide. The globulins, in

0.05 M Tris-HCl buffer, pH 8.2, containing 0.15 M NaCl were treated with 2 ME, to give a final concentration of 0.1 M. After 1 h at room temperature, the globulin was alkylated with an excess of 1.2 molar iodoacetamide. The mixture was kept at 0 °C for 1 h and then dialyzed against the Tris-HCl buffer for 2 days. The YMMH supernatant completely lost its agglutination titer after 2 ME-treatment, suggesting that the supernatant contained IgM antibody.

Preparation of suspensions of dissociated trypanosomes (D-trypanosomes). The trypanosome suspension aggregated by YMMH supernatant at the optimal antigen-antibody ratio was mixed with fresh normal rabbit serum as a source of complement to completely dissociate the aggregated masses. When dissociation was completed, the suspension was mixed with 10 units of CVF to inactivate excess complement. The trypanosomes, designated as D-trypanosomes, were centrifuged at 800 g for 5 min. After centrifugation, suspensions containing varying numbers of the D-trypanosomes in GPB were prepared.

Tissue culture of mouse peritoneal macrophages. Peritoneal exudates of BALB/c mice were induced by i.p. injection of 1 ml of 1% sterile glycogen in Hank's balanced salt solution. After 4 days, the mice were killed rapidly with chloroform and the peritoneal exudates were collected. Peritoneal cells were cultured in Leighton tubes, each containing an 8 × 30 mm coverslip. The cell suspension (1×10^5) in 1 ml of medium consisting of RPMI 1640, 20% calf serum, 50 units/ml of penicillin, and 50 µg/ml of streptomycin was dispensed into each culture tube. The tubes were then allowed to stand at 37 °C for 60 min and were vigorously shaken before withdrawing the medium. The attached cell layer was washed once with 2 ml of the medium and then overlayed with 0.8 ml of the medium for incubation.

Binding test. The culture medium was removed from the macrophage cultures with a sterile pipette and 0.2 ml of *T. gambiense* suspension was added. The culture tube was incubated at 37 °C for 5 min and the coverslip was vigorously washed twice in 2 ml of GPB to remove *T. gambiense* that did not adhere to the macrophages. The coverslips were fixed in methanol for 10 min and stained in Giemsa, and were observed under a microscope (× 400). Binding index (BI) is defined as the number of macrophages with adherent *T. gambiense* × 100/total number of macrophages observed.

Passive transfer. Supernatant of YMMH (0.5 ml) was injected i.p. into recipient mice (BALB/c, 20 g b.wt) one hour before trypanosome challenge. Control mice received an equal volume of P3-X63Ag8-653 myeloma supernatant. To estimate protective ability of the supernatant, trypanosomes (1×10^4) in 0.1 ml GPB were given i.p. to five recipient mice, which were examined for the presence or absence of the parasites every 24 h by wet mount. If no parasites were observed by day 10 after infection, then protection was considered to be complete.

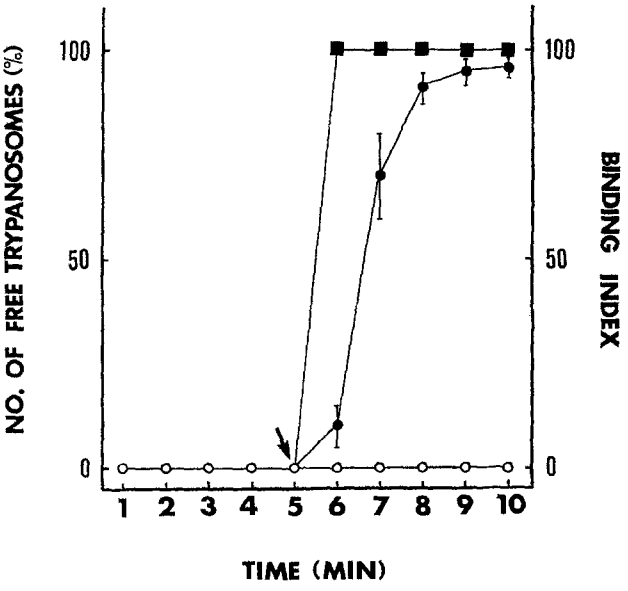
Results

Table 1 summarizes the immune reaction of various trypanosome concentrations mixed with monoclonal IgM mouse antibody, YMMH, without complement. Experiments reported here were done in triplicate. Trypanosomes formed large aggregates and no free trypanosomes were observed in mixtures 1, 2, and 3. These aggregates formed within a few seconds after mixing the trypanosome suspension with supernatant of YMMH. In mixtures 4, 5, and 6, aggregates of trypanosomes were smaller and the number of free trypanosomes increased as antibody ratio increased. In mixtures 7, 8, and 9, trypanosome aggregation was no longer observed in the presence of antibody. It was determined from table 1 that suspension 3 represented the equivalence point, the point at which the antigen-antibody ratio was optimal.

Table 1. Immune responses with different trypanosome-antibody ratios[a]

Experimental number	Number of trypanosomes ($\times 10^8$/0.2 ml)	Supernatant YMMH added (ml)	Clumping	Free trypanosomes (%)
1	20.0	0.1	+	0
2	10.0	0.1	+	0
3	5.0[b]	0.1	+	0
4	2.5	0.1	+	18.5 ± 4.6
5	1.3	0.1	+	67.2 ± 5.7
6	0.16	0.1	+	90.8 ± 8.1
7	0.08	0.1	−[c]	100
8	0.02	0.1	−	100
9	0.002	0.1	−	100

[a] Observations were made 5 min later; [b] Equivalence; [c] The indicated reaction did not occur.



Relationship between the number of *T. gambiense* (■–■) dissociated by complement and the BI value of BALB/c mouse macrophages (●–●) in relation to time in minutes. The parasite suspension was mixed with the supernatant at equivalence. Five min later, fresh normal rabbit serum as the source of complement was added to the mixture (↘) in which trypanosomes formed large aggregates and no free trypanosomes were observed. No free parasite was observed in the control mixture without complement (○–○).

When complement was added to a suspension containing IgM-mediated aggregate masses formed at equivalence point without complement, instant dissociation of the aggregate masses readily occurred (fig. 1). All the aggregated trypanosomes became free within 60 s. These free trypanosomes exhibited vigorous flagellar movement. No lysis of dissociated trypanosomes by complement was observed. As soon as the clumped trypanosomes became free, they attached to cultured macrophages (fig.). However, no dissociation of IgM-mediated clumping masses was observed when complement inactivated with CVF was added to the suspension.

On the other hand, when supernatant of YMMH was added to the trypanosome suspension at equivalence point in the presence of complement, initiation of trypanosome aggregation was completely inhibited and only free trypanosomes were observed. When the supernatant of YMMH was added to the parasite suspension at equivalence point in the presence of the fresh serum previously inactivated with CVF, trypanosomes formed large aggregate masses with no unbound organisms. When a predetermined concentration of CVF was added, dissociation of trypanosomes, initiated in the presence of fresh sera, was immediately terminated, but reaggregation of the trypanosomes that had been dissociated did not occur.

Table 2 summarizes aggregation of D-trypanosomes in suspensions of various concentrations remixed with fresh supernatant of YMMH without complement. Experiments reported here were done in triplicate. D-trypanosomes formed large aggregates, with no free D-trypanosomes, in mixtures 1, 2, and 3. Aggregates of D-trypanosomes in mixtures 4 and 5 were smaller and the number of free D-trypanosomes increased as the IgM antibody ratio increased. The data from table 2 show that the D-trypanosomes:fresh IgM antibody ratio was optimal in mixture 3.

Table 2. Immune responses between D-trypanosomes and the supernatant YMMH in different antigen-antibody ratios[a]

Experimental number	Number of D-trypanosomes ($\times 10^8$/0.2 ml)	Supernatant YMMH added (ml)	Clumping	Free D-trypanosomes (%)
1	20.0	0.1	+	0
2	10.0	0.1	+	0
3	5.0[b]	0.1	+	0
4	2.5	0.1	+	34.5 ± 4.9
5	1.3	0.1	+	81.7 ± 6.7

[a] Observations were made 5 min later; [b] Equivalence.

Table 3. Aggregation reaction between presensitized trypanosomes and untreated ones[a]

	Presensitized trypanosomes ($\times 10^8$/0.2 ml)	Number of untreated trypanosomes added ($\times 10^8$/0.2 ml)	Clumping
IgM-Binding:	2	1	+
	1	1	+
IgM-Complement-Binding:	2	1	–[b]
	1	1	–

[a] Observations were made 5 min later; [b] The indicated reaction did not occur.

Table 3 summarizes the aggregation reaction between presensitized trypanosomes with or without complement and new untreated ones. Experiments reported here were also done in triplicate. Aggregate masses formed instantly in the mixture of IgM-binding trypanosomes and untreated new trypanosomes. However, no aggregate masses were observed in the mixture of IgM-complement-binding trypanosomes and new untreated ones.

Passive transfer of immunity was achieved using supernatants of YMMH cultures, and it was effective for complete protection against trypanosomes. Mice given the supernatant i.p. were protected against infection with as many as 1×10^4 trypanosomes. No control mice were protected against trypanosome infection. They showed parasitemia on day 2 after infection, and died on day 4.

Discussion

Immune complexes that form after antigen-antibody reaction between soluble antigen and the homologous antibody are well known to activate the complement system. Conversely, complement activation markedly influences the fate of the immune complex; aggregates of immune complexes are solubilized by the activated complement system[10–12]. Such a solubilized immune complex is composed of antigen, antibody and complement[10]. The solubilization mechanism is still obscure. Factors such as steric hindrance of antibody by complement, and physicochemical properties such as avidity, affinity and epitope difference, are suggested to be operative in the solubilization process.

The dissociation of antibody-mediated trypanosome masses by complement and inhibition of antibody-mediated aggregation in the presence of complement may be essentially similar to the solubilization of aggregates of soluble antigen and antiserum by complement. Recently, it was reported that the dissociation of bivalent antibody-mediated clumps was associated with the functional change of the nature of the bivalent antibody into a univalent one in the presence of complement[3,4].

When trypanosomes came into contact with mAb. IgM at equivalence, an aggregation reaction instantly occurred, resulting in the formation of clumped masses with no unbound organisms. These clumped trypanosomes were dissociated by the addition of complement to become free, and free trypanosomes easily adhered to the surface of the cultured peritoneal macrophages (fig.) through immune complexes formed on their surface. Lysis of dissociated trypanosomes by complement did not occur, because they had substances which inhibited the cytolytic activities of complement[13,14]. Since trypanosomes move freely by means of their flagella, an unexpected event in an aggregation reaction in which antibody acts as an adhesive agent between trypanosomes can be readily detected by observing aggregated trypanosome masses under the microscope.

Judging from tables 1 and 2, dissociation of the IgM antibody-mediated clumps of trypanosomes in the equivalence area released a large amount of antigens previously bound to the antibody, and released antigens were capable of combining with new antibodies again, resulting in the observed clumping of the D-trypanosomes indicated in table 2. Since IgM antibody consists of five 7s-subunits, each of which has two antigen binding sites (ABS)[6], all ABSs of the monoclonal IgM antibody would be expected to combine with the surface epitope of *T. gambiense* at equivalence point to form immunocomplexes, resulting in agglutination. It was conjectured that in the presence of fresh serum as the source of complement, changes would occur in the IgM antibody. Such changes were expected to lead to further changes: one ABS of the antibody would continue to form an immunocomplex with the antigen, but the other ABSs would no longer bind to the antigen (table 3), resulting in the dissociation of clumped masses or inhibition of aggregation.

IgM antibodies on trypanosomes presensitized in the extreme antibody excess area lost their capacity to bind to fresh surface antigens after the treatment with fresh serum (table 3). Thus, this release of a large amount of antigen was associated with the loss of binding ability in many binding sites of every IgM antibody. So, the optimal antigen-IgM antibody ratio in the absence of complement became a condition of extreme antigen excess in the presence of complement. Incidentally, IgG-mediated aggregates formed in the equivalence area without complement were also dissociated by complement. This dissociation released approximately 50% of previously bound surface antigens[3,4], suggesting that the bivalent antibody was made functionally univalent by complement. Complement binding to IgG molecules may occur on the Fd region[15].

IgM antibody is composed of a joining chain connecting five 7s-subunits in the central portion[16]. The function of the joining chain is not known in detail. Formation of IgM antibody-mediated trypanosome aggregates in the presence of complement was completely inhibited, indicating that an initial stimulus, making the antibody functionally univalent in nature, passes promptly from a subunit which first binds to the surface antigen, through the joining chain, to the other subunits.

Trypanosomes were effectively removed from the circulation of experimentally infected mice by passive transfer of monoclonal IgM antibodies to their surface antigen. Events involving the dissociation of IgM antibody-mediated aggregates of trypanosomes by the complement system seem to be operative to clear the infected host of *T. gambiense* in vivo, because the antibody and complement forming immune complex promoted immunophagocytosis of cultured peritoneal macrophages, which is important in eliminating trypanosomes[17,18].

The roles of IgG-immunoglobulin and complement in opsonization have been studied extensively[19,20]. Fixation of the target to the surface of the phagocytic cell is the first phase of the phagocytic process[21]. Complement plays a vital function in this fixation[22]. It is frequently accomplished by the interaction of complement residing on the target with special receptors for Clq and C3 degradation fragments C3b, iC3b and C3dg on the phagocytic cells. The synergistic effect of IgG immunoglobulin and C3b in phagocytosis is thought to arise from a concomitant binding of FcR (IgG receptor) and CR1 (C3b receptor) on phagocytes. However, IgM-immunoglobulin is less well studied in the role it plays in immunophagocytosis.

Various biological activities in which antibody is involved are largely due to the biological activities of complement activated by the immune complex. Therefore, the intensity of these biological activities of the antibody is affected by the number of complement molecules activated by the complex. In this study, the multivalent IgM antibody became functionally univalent upon complement activation, with one antibody binding to one antigen on the cell surface of a trypanosome. Trypanosomes are known to have a great number of surface antigens which consist of glycoproteins[23]. Therefore, a large number of univalent antibodies may be mobilized for the formation of immunocomplexes and subsequent activation of complement. For clearance of trypanosomes from infected host animals, IgM antibody is known to be more effective than IgG antibody[4]. These results support this contention, considering that an IgM antibody can bind 5 more complement molecules than a single IgG antibody can.

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