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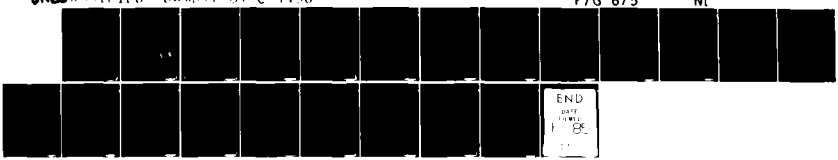

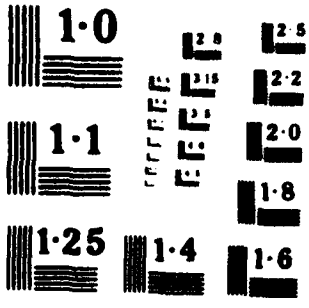
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Characterization of Antibody-Dependent Killing of Trypanosomes by Macrophages

Annual Summary Report

David L. Rosenstreich, M.D. and
Hellen C. Greenblatt, Ph.D.

April, 1983

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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ABSTRACT

In the presence of antibody, macrophages bind trypanosomes in vitro. The cellular and humoral components of this assay have been studied and defined. In general, such antibody must be specific for the trypanosome variant. However, WRATat-14 and WRATat-15 variants, obtained after vector transmission of WRATat-1.1, mediated of WRATat-1 trypanosomes to macrophages. Using a large panel of anti-WRATat-1.1 specific monoclonal antibodies, a close correlation was found between the ability to mediate macrophage binding in vitro, and in vivo trypanosome neutralization. The relative efficacy of various immunoglobulin subclasses was analyzed. Monoclonal antibodies of all subclasses were able to mediate trypanosome binding by macrophages. However, when polyvalent anti-WRATat-300 (Wellcome) serum from a rabbit infected 4 weeks was fractionated into isotype specific-immunoglobulins, only the cold-precipitable portion of IgM was functional. The role of complement was also analyzed. It was confirmed that complement enhances macrophages binding of trypanosomes in the presence of limiting dilutions of antiserum. There was no species specificity in the action of complement since dog, human, guinea pig and mouse sera could be used as complement sources. In the presence of C4 deficient complement, or sera depleted of C2 and Factor B, binding was substantially decreased as compared to that found in normal serum. Binding in C5 deficient sera was not effected. These findings suggest that complement mediated binding of trypanosomes by antibody required components of both pathways, but did not require terminal components.

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20. SUMMARY

In the presence of antibody, macrophages bind trypanosomes in vitro. The cellular and humoral components of this assay have been studied and defined. In general, such antibody must be specific for the trypanosome variant. However, MRATat-14 and MRATat-15 variants, obtained after vector transmission of MRATat-1.1, mediated binding of MRATat-1 trypanosomes to macrophages. Using a large panel of anti-MRATat-1.1 specific monoclonal antibodies, a close correlation was found between the ability to mediate macrophage binding in vitro, and in vivo trypanosome neutralization. The relative efficacy of various immunoglobulin subclasses was analyzed. Monoclonal antibodies of all subclasses were able to mediate trypanosome binding by macrophages. However, when polyvalent anti-MRATat-300 (Wellcome) serum from a rabbit infected 4 weeks was fractionated into isotype specific-immunoblobulins, only the cold-precipitable portion of IgM was functional. The role of complement was also analyzed. It was confirmed that complement enhances macrophage binding of trypanosomes in the presence of limiting dilutions of antiserum. There was no species specificity in the action of complement since dog, human, guinea pig and mouse sera could be used as complement sources. In the presence of C4 deficient complement, or sera depleted of C2 and Factor B, binding was substantially decreased as compared to that found in normal serum. Binding in C5 deficient sera was not effected. These findings suggest that complement mediated binding of trypanosomes by antibody required components of both pathways, but did not require terminal components.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

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Characterization of Antibody-Dependent Killing of Trypanosomes by Macrophages. Contract No. DAMD17-81-C-1196

ANNUAL REPORT

This contract was initiated September 1981 to characterize the phenomenon of macrophage binding of opsonized T. rhodesiense (Greenblatt et al 1983). The contract had the following goals:

(a) Determine the requirement for variant specific antibody.
(b) Analyze the ability of monoclonal antibodies specific for discrete trypanosome antigenic determinants to mediate macrophage-trypanosome binding.
(c) Determine the immunoglobulin class dependence of this binding.
(d) Analyze the effect of specific complement pathways and components.
(e) Characterize the macrophage functions and receptors active in this system.

Progress was made in the following areas:

(a) Determination of the requirement for variant specific antibody

Injection of a clone of trypanosomes into mice will result in an antiserum that is specific for that clone (Campbell, et al 1979). Antibody-mediated, complement dependent killing of trypanosomes in vitro requires variant-specific antibody (Diggs and Toussaint, personal communication) as do other antibody-dependent immune responses directed against trypanosomes. Some investigators have demonstrated that only variant-specific antibody will mediate the binding of T. gambiense to macrophages (Takayanagi and Nakatake, 1977). However, Cook (1981) found that antigen-specificity of rabbit immune sera was not a factor in the binding of T. brucei Etat 4 or T. brucei 427 to rabbit peritoneal cells. It was therefore necessary to test the need for specificity of antibody in mediating the attachment and internalization of trypanosomes to macrophages.

The results of a series of experiments to determine whether trypanosome binding to murine macrophages required variant-specific antibody are summarized in Table I. Normal mouse sera were unable to mediate binding of any trypanosomes, regardless of which trypanosome variant was used. Rabbit anti-MRATat-300 (Wellcome) and the monoclonal antibody directed against MRATat-1 could only mediate binding of their respective trypanosomes. Conclusions: These data suggested that antibody directed against a specific variant type was required to mediate binding of these parasites to macrophages.

There is also evidence for cross-reactivity between antibodies. MRATat-14 and MRATat-15 are two variant antigen types obtained after vector transmission of MRATat-1.1 (Esser et al 1982). Sera from three monkeys injected with these new variants were able to mediate binding of MRATat-1 trypanosomes to mouse resident peritoneal macrophages. Such cross reactivity between various trypanosome strains may be useful for the development of new diagnostic assays since only a primary antigenic type and/or antibody directed against the type, would be required.

(b) Analysis of the ability of monoclonal antibodies specific for discrete trypanosome antigenic determinants to mediate macrophage-trypanosome binding

Klaus Esser (WRAIR, Washington, D.C.), has developed about 40 hybridomas by fusing WRATat-1.1 spleen cells from infected mice to immunoglobulin-secreting myeloma lines (Sacks, et al 1982). These hybridomas generate monoclonal antibodies which react with variant antigen types of trypanosomes. The majority of these products are capable of neutralizing parasite infectivity (Table II).

Each of the monoclonal antibodies were screened over a large range of concentrations, and in the presence or absence of enhancing levels of complement in the macrophage assay. A careful microscopic analysis was then performed for each individual monoclonal antibody and the percentage of macrophages binding trypanosomes was calculated.

Table II lists the monoclonal antibodies assayed, their ability to mediate binding of trypanosomes to macrophages in vitro, and their ability to neutralize infection in vivo. There was a close positive correlation between the ability to mediate binding of WRATat-1 trypanosomes to resident peritoneal cells and the ability of the monoclonal antibody to opsonize trypanosomes for in vivo neutralization; 91% of the monoclonal antibodies could both mediate binding and neutralize in vivo.

Two IgG1 monoclonal antibodies, 6.7H11 and 16.3F1.4, were able to mediate binding especially when enhanced by the presence of complement; they were not however protective in vivo. (Klaus Esser, personal communication). Monoclonal antibody 15.2H5.1 presented difficulties suggesting that two separate lots, with different potencies may be involved. The first lot of 15.2H5.1 tested was unable to mediate binding. However, a second lot produced good binding. When neutralization studies were carried out with 15.2H5.1 results were negative or ambivalent. Currently it is typed as a monoclonal antibody incapable of neutralization in vivo; however, if different lots were assayed this negative designation might be reversed.

Monoclonal antibodies of subclasses tested including: IgM, IgG1, IgG2a, and IgG2b, were able to mediate binding of trypanosomes to macrophages. There did not appear to be any correlation between the immunoglobulin class and its functional activity.

(c) Determination of the immunoglobulin class dependence of binding

Dr. Jeenan Tseung of WRAIR, Washington, D.C. provided us with antibody subclasses of rabbit anti-WRATat-300 (Wellcome) strain of T. rhodesiense. Serum was collected from an infected rabbit over a 4 week period, and antibody subclasses prepared by sequential ammonium sulfate precipitation (35-40%), cold precipitation, ion exchange chromatography on DEAE-cellulose, and affinity chromatography on immobilized staphylococcal protein A.

These fractions were demonstrated to be isotype specific IgM, IgG2a, and IgG2b, and were demonstrated to have no contamination with IgA. The cold-precipitable portion of IgM was able to mediate binding of trypanosomes to 80% of the macrophages both in the presence or absence of complement

(Table III). None of the other fractions were able to promote any attachment. These data suggest that only the IgM fraction of rabbit anti-WRATat-300 was functional in this assay. Other reports have indicated that antibodies of both IgM and IgG specific to trypanosomes appear within 3 weeks of infection (Seed et al 1969, Musoke et al 1981, Cook 1981). However, Musoke et al (1981) determined that along with IgM, the IgG1 isotype, not the IgG2, was responsible for clearance of parasites in cattle which is consistent with our in vitro findings. We did not have any IgG1 fractions to assay so the question of IgG1 specificity for the parasite cannot be resolved. This data differs from our finding using the monoclonal antibodies (section b, above). In those experiments, it was found that monoclonal antibodies of all isotypes were capable of mediating binding to macrophages. Such diversity may reflect the nature of the monoclonal antibody generating system rather than a situation found "in nature". Whether only specific Ig subclasses are functional against trypanosomes is an important question, and will require additional experimentation for its resolution.

(d) The effect of specific complement pathways and components

Introduction

The complement cascade of serum consists of functionally inactive precursor molecules. Activation is the term designated for the process which permits these proteins to cooperate in a specific sequence. Activation of C3 is one of the central events of the complement cascade and occurs via the classical and/or alternative pathways. Enzymes generated during the cascade cleave the C3 molecule, producing several biologically active molecules (C3a, C3b and C3d), and initiating the terminal complement cascade (activation of C5-C9).

The components of the classical complement system and the late acting terminal proteins are designated numerically as C1, C4, C2, C3, C5, C6, C7, C8, and C9. The classical pathway is initiated by an antibody-antigen reaction which activates the sequence, C1, C4, C2, and finally C3. Cleavage terminal sequence is triggered (either via the classic or alternative pathways) lysis of the particle binding complement may occur.

The second pathway by which C3 can be cleaved and the terminal pathway (C5-C9) initiated, is the alternative pathway. When this system is functional, the classic pathway may be by-passed resulting in little or no consumption of C1, C4, or C2. The alternative pathway functions by activating Factor B in the presence of magnesium ions. Cleavage of B results in an enzyme that is capable of activating C3 which in turn will cleave C5-C9 (the terminal sequence).

The Fc portion of antigen-antibody complexes bind to specific receptors on macrophages. The Fc receptors of macrophages are triggered to initiate phagocytosis of IgG-coated and perhaps IgM-coated (Silverstein et al 1977, Roubin and Zolla-Pazner 1979) particles. These receptors also appear to have an immunoregulatory role (Morgan et al 1983). There are other sets of receptors on macrophages that bind fragments of C3. Such binding, via C3b receptors, is thought to enhance particle ingestion via Fc receptors (Ehlenberger and Nussenzweig, 1977). Also under specific conditions, particles coated only with C3b will be readily phagocytized (Griffin and

Mullinax 1981).

1) Role of complement in enhanced binding of trypanosomes to macrophages in the presence of limiting dilutions of antibody

In previous work (Greenblatt et al 1983) it was demonstrated that immune serum whic had been heat-treated to inactivate complement, mediated progressively lower percentages of trypanosome binding to macrophages as the antiserum was diluted to 10^{-1} to 40^{-1}. Upon the addition of rat complement to these samples, macrophages again bound high numbers of trypanosomes. These data suggested that in the presence of limiting dilutions of antiserum, rat complement enhances the binding of trypanosomes to macrophages.

Therefore, in order to further investigate the role of complement in the increased binding of trypanosomes to peritoneal macrophages, the complement that is normally present in serum had to be inactivated. Heat-inactivation of serum at 53°C for 90 min destroys many complement proteins and inactivates the complement pathway. Sera were treated in this manner and tested for their ability to mediate binding of trypanosomes. In the presence of limiting dilutions of heat inactivated sera only 22% of the macrophages bound trypanosomes (Table IV). When these sera were supplemented with rat complement, 58% of the macrophages bound parasites. Guinea pig, dog, human, and mouse sera could also be used as complement sources (Table IV). These findings indicate that complement enhances macrophage binding of trypanosomes in the presence of limiting dilutions of antiserum. In addition, there is no species specificity in this action of complement.

2) Role of terminal, classical and alternative pathway components

The requirement for specific complement components in the binding assay was determined by testing sera that were lacking individual components.

There has been demonstrated a requirement for antibody, alternative pathway and terminal components for the killing of T. rhodesiense in vitro (Flemmings and Diggs 1978). Therefore it was possible that the killing of parasites was mediated by macrophage complement components released and activated by parasites at the surface of the macrophages.

Lysis at the macrophage surface has been shown to be triggered in other systems by specific IgG fractions (Ralph 1980; Walker 1977). Lysis cascades typically require terminal components including C5. Mouse sera deficient in C5 was utilized to determine the need for terminal complement components in mediating killing of trypanosomes by macrophages. In the presence of normal mouse serum, 60% of the macrophages bound trypanosomes, as compared to 79% binding in the presence of C5 deficient serum (Table IV).

Also, guinea pig serum deficient in C4 components of the classical pathway (C4D) was compared to normal guinea pig serum for its ability to enhance uptake under conditions of limited levels of antibody. This serum only partially restored the binding activity of antibody (Table IV). In the presence of C4D serum only 44% of the macrophages bound trypanosomes compared with 68% binding in the presence of intact guinea pig serum (Table IV).

Treatment of serum at 50°C for 20 min partially decreases levels of C2

(of the classical pathway) and destroys Factor B (of the alternative pathway). When such heat-treated complement was added to heat-inactivated serum, binding activity was only partially restored (from 22% to 41%) (Table IV).

Conclusions: Complement mediated binding of trypanosomes by antibody requires components of both pathways, but does not require terminal components. The most plausible interpretation is that such enhancement is mediated via macrophage C3b receptors.

(e) Characterization of the macrophage functions and receptors active in this system

We have begun to screen a number of normal and mutant mouse macrophage cell lines for their ability to bind trypanosomes. Currently, we are determining the growth characteristics and adherence characteristics of these lines. Several of these lines exhibit stable, well defined abnormalities (Table V). Using mutant cell lines which lack specific surface receptors or are metabolically abnormal may provide insight into the mechanisms by which macrophages clear trypanosomes.

TABLE I

Effect of monoclonal antibody, and sera obtained from infected mice and monkeys on the binding of variant trypanosomes

Serum	WRATat-300 (Wellcome)	WRATat-1	WRATat-186 (EATRO 1886)
Normal mouse sera	-	-	-
Rabbit anti-WRATat-300	+	-	-
Monoclonal 2.187.1 (anti-WRATat-1)	-	+	-
Monkey pre-bleed		-	
Monkey anti-WRATat-14		+	
Monkey anti-WRATat-15		+	

TABLE II

Ability of monoclonals to neutralize *in vivo* and mediate *in vitro* binding to macrophages

	In vivo Neutralization	Macrophage Binding	Class
31.4D1.6	0	0	IgM
32.1A5.5	+	+	IgM
32.2A1.1	+	+	IgM
32.2A4.1	+	+	IgM
32.3F1.6	+	+	IgM
2.1B7.1		+	+IgG1
*6.7H11		0	+IgG1
6.8C1.2		+	+IgG1
6.11D5		+	+IgG1
16.2A12.2		+	+IgG1
*16.3F1.4		0	+IgG1
2.1D8.1		0	0IgG2a
12.3D3.4		+	+IgG2a
12.4F3.1		+	+IgG2a
16.1A5.3		+/-	+IgG2a
16.1A8.1		+	+IgG2a
32.1A3.1		0	0IgG2a
32.1B5.1		+	+IgG2a
4.1G9.1		0	0IgG2b
12.2E7.2		+	+IgG3
*15.2H5.1		-	+IgG3
31.4G5.1		0	0IgG3
32.3B1.5		0	0IgG3
32.4C1.5		0	0IgG3
32.3B12.1		+	+

*Indicates discrepancy between *in vivo* and *in vitro* findings.

TABLE III

Effect of fractions isolated from rabbit anti-trypanosome serum to mediate binding of trypanosomes

	% Binding of Trypanosomes to Macrophage	
	Cold Precipitable	Non-cold Precipitable
Hyperimmune rabbit serum (83%)		
IgM		
without complement	85	0
with complement	76	0
IgG2a		
with or without complement	0	0
IgG2b		
with or without complement	0	0

TABLE IV

Ability of complement deficient and intact sera to enhance binding of trypanosomes to macrophages under conditions of limited amounts of heat-activated[1] anti-trypanosomal antibody

Treated Rat Complement	% Attachment
53°C/90 min[1]	22 ± 6.5
50°C/20 min[2]	41 ± 2.8
Media	21 ± 1.6
Intact Sera	
rat	58 ± 5.6
guinea pig	68 ± 2.0
dog	68 ± 13
human	54 ± 1.5
mouse	69 ± 15
Complement Deficient Sera	
C4 deficient guinea pig	44 ± 4.5
C5 deficient mouse	79 ± 7

[1]Anti-trypanosome antibody was heat-inactivated at 53°C/90 min to disrupt the complement cascade.

[2]Complement was heated at 50°C for 20 min to destroy Factor B and C2.

TABLE V

Mouse macrophage lines to be tested for trypanosome binding

Line	Functional Characteristics
J774.2	Fc mediated phagocytosis
J774.1.11	Defective in IgG2a phagocytosis normal binding of monomeric IgG2a
J774.3.4	Depressed levels of IgG2a and IgG2b phagocytosis
$CTRM_1$	Lacks CAMP dependent protein kinase

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