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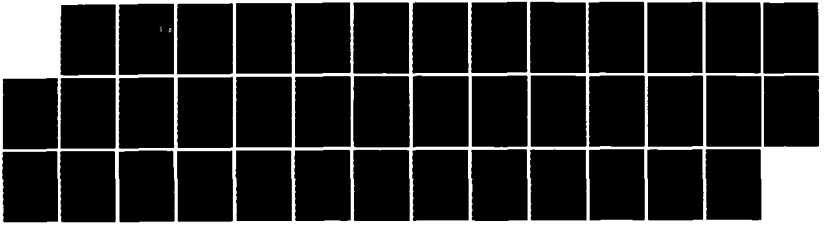

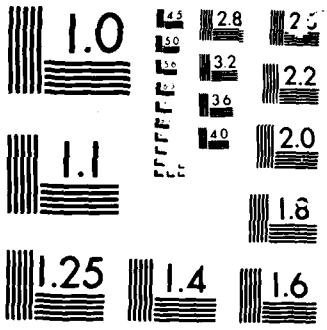
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Characterization of Antibody-Dependent Killing of Trypanosomes by Macrophages.

Final Report

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Hellen C. Greenblatt, Ph.D.

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In the presence of specific antibody, murine peritoneal macrophages and macrophage-like cell lines bind Trypanosoma rhodesiense parasites in vitro. There is a high correlation between the ability of these products to neutralize trypanosome infection in vivo and mediate binding of trypanosomes to macrophages in vitro and a high coincidence between function in the macrophage-binding assay and the ability of monoclonal antibody to bind trypanosomes in an indirect immunofluorescence assay. Trypanosomes do not actively penetrate macrophages, but instead are phagocytized by the macrophages. The C3 component of complement is an important co-factor in IgG mediated binding and uptake of trypanosomes by macrophages. There is a partial genetic regulation of this phenomenon. Normal peritoneal macrophages from C57BL/6, BALB/c, and C3H/HeJ mice do not differ in their trypanosome-binding ability. However, 10 days post-infection, cells from genetically susceptible, BALB/c mice bound more trypansomes than did those from infected genetically resistant, C57BL/6 mice. Both murine (P388D1 and J774.2) and human (BB, CG, CS, and DM)

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SUMMARY

In the presence of specific antibody, murine peritoneal macrophages and macrophage-like cell lines bind Trypanosoma rhodesiense parasites in vitro. Using a large bank of anti-WRATat 1.1 specific monoclonal antibodies, developed by Klaus Esser, Walter Reed Army Institute of Research, Washington, D.C., a high correlation was found between the ability of these products to neutralize trypanosome infection in vivo and mediate binding of trypanosomes to macrophages in vitro. There was also a high coincidence between function in the macrophage-binding assay and the ability of a monoclonal antibody to bind trypanosomes in an indirect immunofluorescence assay, (Hall and Esser, 1984). The mechanism by which trypanosomes penetrate macrophages was also investigated. The data suggest that these organisms do not actively penetrate macrophages, but instead are phagocytized by the macrophages. The mechanism underlying the enhancement of binding by fresh serum was analyzed, and the C3 component of complement was found to be important. Possible genetic control of macrophage function was also investigated. Normal peritoneal macrophages from C57BL/6, BALB/c, and C3H/HeJ mice do not differ in their trypanosome-binding ability. However, 10 days post-infection, cells from genetically susceptible, BALB/c mice bound more trypanosomes than did those from infected genetically resistant, infected C57BL/6 mice. A number of macrophage cell lines were analyzed for their trypanosome binding ability. Both murine (P388D1 and J774.2) and human (BB, CG, CS, and DM) macrophage cell lines were found to be capable of binding trypanosomes in the presence of specific antisera.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. 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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FINAL REPORT

For the period 15 September 1981 to 14 November 1983.

This contract was initiated September 1981 to characterize the phenomenon of macrophage binding of opsonized T. rhodesiense organisms in vitro (Greenblatt et al 1983).

The contract had the following goals:

1. To determine the requirement for T. rhodesiense variant specific antibody in the assay,

2. To analyze the ability of hybridoma-derived T. rhodesiense special monoclonal antibodies to mediate trypansome binding in vitro,

3. To compare the ability of monoclonal antibodies which mediate binding of trypanosomes to macrophages in vitro with their ability to neutralize trypanosomes in vivo,

4. To determine the immunoglobulin class dependence of binding in vitro.

5. To analyze the effect of specific complement components in enhancing macrophage binding of trypanosomes,

6. To characterize macrophage functions and receptors active in this system,

7. To determine whether macrophages from mice which differ greatly in their natural resistance to infection with T. rhodesiense, exhibit different capabilities in their binding and/or killing of trypanosomes in vitro, and

8. To test the trypanosome binding ability of murine and human macrophage cell line cells.

A) DESCRIPTION OF MACROPHAGE BINDING ASSAY

In the presence of trypanosome-specific antibodies, cultured peritoneal macrophages or macrophage cell lines will bind and internalize Trypanosoma rhodesiense (Greenblatt, et al. 1983). The findings of Mac Askill et.al. (1980) also suggest that this in vitro system reflects a physiological pathway for trypanosome clearance.

In this assay, monolayers of resident peritoneal macrophages derived from C57BL/6 mice are cultured in tissue culture chamber slides (Lab Tek, Miles, Elkhart, IN) at 37°C in a 9% CO_2 atmosphere for 6-18h. At the end of the incubation period non-adherent cells are removed, fresh medium placed onto the remaining cells, and the slides incubated for a total period of 48-72h.

The monolayers are then exposed to trypanosomes in the presence of specific antibody (generated in rabbits or rats exposed to the appropriate trypanosome strain) or to WRATat 1-specific monoclonal antibodies developed by K. Esser (Walter Reed Army Institute of Research (WRAIR) Washington, D.C). After incubation of cultures with antibody and trypanosomes for 30 min. the cultures are washed, fixed with Giemsa stain, and then assayed by light microscopy. One hundred cells are counted and their binding and/or internalization of trypanosomes is recorded.

B) 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 serum was unable to mediate binding of trypanosomes, regardless of which trypanosome variant was used. Rabbit anti-WRATat-300 (Wellcome) and the monoclonal antibody directed against WRATat-1 could only mediate binding of their respective trypanosomes.

There is also evidence for cross-reactivity between antibodies. WRATat-14 and WRATat-15 are two variant antigen types obtained after vector transmission of WRATat-1.1 (Esser et al 1982). Sera from three monkeys injected with these new variants were able to mediate binding of WRATat-1 trypanosomes to mouse resident peritoneal macrophages. The most likely explanation of this result is that WRAT at-14 and 15 share surface antigens in common with WRAT at-1.1.

Overall, the results of this series of experiments are consistent with the hypothesis, that antibody directed against a specific variant type was required to mediate binding of these parasites to macrophages.

C) ANALYSIS OF THE ABILITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR DISCRETE TRYPANOSOME ANTIGENIC DETERMINANTS TO MEDIATE MACROPHAGE TRYPANOSOME BINDING IN VITRO

Klaus Esser of WRAIR, Washington, D.C., has fused immunoglobulin-secreting P3/X63-Ag8 or X63-Ag8.653 (Kearney, et al. 1979) myeloma cells lines and spleen cells from WRATat-1.1 infected mice. These fusions resulted in a

TABLE I

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

	Trypanosome Variant		
Serum	WRATat-300 (Wellcome)	WRATat-1	WRATat-186 (EATRO 1886)
Normal mouse sera	-	-	-
Rabbit anti-WRATat-300	+	-	-
Monoclonal 2.1B7.1 (anti-WRATat-1)	-	+	-
Monkey pre-bleed		-	
Monkey anti-WRATat-14		+	
Monkey anti-WRATat-15		+	

large number of hybridomas capable of reacting with, and neutralizing specific variants of parasites.

A large number of these monoclonal antibodies were provided to this laboratory for screening in our in vitro macrophage binding assay. The primary goal was to correlate in vitro binding to in vivo neutralization results, and determine whether the in vitro assay could replace in vivo neutralization tests or indirect fluorescence tests.

Neutralization of parasites, as carried out by the laboratory of K. Esser, (Hall and Esser, 1984) consisted of incubating parasites with the variant specific monoclonal antibody for 10 min and then injecting the suspension interperitoneally into the host mouse. Parasitemia was monitored by tail bleeding. The absence of parasites during the expected first wave of infection and throughout a 30 d period was considered proof that the tested antibody was capable of neutralizing a parasitic infection with a specific variant.

The ability of a variety of monoclonal antibodies to mediate in vitro binding of trypanosomes in the presence of exogenously added rat complement was determined (Table II). These in vitro results with each monoclonal antibody were compared with the in vivo neutralization results supplied to us by K. Esser.

Of the monoclonal antibodies assayed, 21.9% (32) were IgM, 31.3% (32) of the IgG1 subclass, 21.9% (32) of the IgG2a subclass, and 18.8% (6) of the IgG3 subclass. IgG2b and IgA each represented 3.1% of the total (or 1 monoclonal antibody each).

71.4% (5/7) of the IgM monoclonal antibodies mediated macrophage binding of trypanosomes (i.e., more than 12% of the macrophages bound trypanosomes). 100% of the IgG1 monoclonal antibodies (10/10) and 71.4% (5/7) of the IgG2a

TABLE II

Comparison of the ability of monoclonal antibodies to neutralize trypanosomes *in vivo* and mediate *in vitro* binding to macrophages.

Monoclonal Antibody	*In vivo* Neutralization**	Percent Macrophage Binding	Ig Class
31.4D1.6	0	9***	IgM
32.1A5.2	+	36	IgM
32.2A1.1	+	89	IgM
32.2A4.1	+	87	IgM
32.2A6.1	0	0	IgM
32.3F1.1	+	68	IgM
32.4G10	+	53	IgM
2.1B7.1	+	35	IgG1
* 6.7H11	0	56	IgG1
6.8C1.2	+	92	IgG1
* 6.10A4.1	0	51	IgG1
* 6.10F11.1	0	40	IgG1
* 6.11B4.1	0	58	IgG1
6.11D5	+	87	IgG1
6.12E4	+	67	IgG1
16.2A12.2	+	87	IgG1
*16.3F1.4	0	43	IgG1
2.1D8.1	0	6	IgG2a
12.3D3.4	+	79	IgG2a
12.4F3.1	+	69	IgG2a
16.1A5.3	+	92	IgG2a
16.1A8.1	+	62	IgG2a
32.1A3.1	0	3	IgG2a
32.1B5.1	+	73	IgG2a
4.1G9.1	0	3	IgG2b
12.2E7.2	+	80	IgG3
*15.2H5.1	0	41	IgG3
31.4G5.1	0	9	IgG3
32.3B1.5.1	0	10	IgG3
32.4C1.5.1	0	6	IgG3
32.3B12.1	+	86	IgG3
6.11A9	+	15	IgA

* Indicates discrepancy between *in vivo* and *in vitro* findings.
** Data supplied by K. Esser (WRAIR).
*** Less than 11% binding indicates negative binding.

antibodies mediated binding of trypanosomes to macrophages. Only 50% (3/6) of the IgG3 monoclonal antibodies were active in this assay. The one IgG2b antibody tested was inactive, and the one IgA antibody tested had only weak activity.

Within all immunoglobulin classes and subclasses there were monoclonal antibodies which mediated binding of trypanosomes to macrophages. Work by Hall and Esser (1984), confirms that humoral immunity to WRATat1 trypanosomes is associated with all classes of antibody.

Table II also compares the ability of monoclonal antibody to successfully neutralize in vivo, and their ability to mediate binding of parasites to macrophages in vitro.

There was 81.3% (26/32) coincidence between the results of neutralization by a monoclonal antibody and its capacity to bind parasites to macrophages. There were 6 monoclonal antibodies in which the in vitro binding assay was positive, but the in vivo neutralization test was negative. No antibodies neutralized in vivo but failed to mediate in vitro binding. 5 of the discrepant antibodies were of the IgG_1, subclass and one was an IgG3.

Hall and Esser (1984) described 3 morphological reactions when indirect fluorescence assays were performed with monoclonal antibody and WRATat-1 organisms. These were: 1) uniform, along the entire surface of the parasite, 2) focused about the region of the flagellar pocket and 3) no binding.

Four of the six antibodies that failed to neutralize in vivo, but mediated in vitro binding, bound to the region of the flagellar pocket (Table III). The remaining two antibodies did not produce detectable binding by the flourescent assay.

TABLE III

Monoclonal Antibodies (Mab) that can mediate *in vitro* binding to macrophages but are unable to neutralize infections *in vivo*.

Monoclonal Antibody	Neutralization	% macrophage binding	Pattern Binding in Flourescent Assay
6.10A4.1	0	51	Flagellar (pocket)
6.10F11.1	0	40	Flagellar (pocket)
6.11B4.1	0	58	Flagellar (pocket)
16.3F1.4	0	43	Flagellar (pocket)
6.7H11	0	56	none
15.2H5.1	0	41	none

* Hall & Esser (1984).

D) ANALYSIS OF THE ACTIVITY OF MONOCLONAL ANTIBODIES IN VITRO

The ability of monoclonal antibodies to promote phagocytosis (in contrast to only external binding) of trypanosomes was also analyzed. There was a variation in the handling of trypanosomes after they were bound. For example, 31.4D1-6.1 was a non-functional monoclonal antibody of the IgM subclass (Tables I and II). It promoted neither attachment nor internalization of trypanosomes. Another monoclonal antibody of the IgM class, 32.2A1.1 mediated binding of parasites to 96% of the macrophages of which all had evidence of internalized trypanosomes. In contrast, monoclonal antibody 15.2H5.1 (IgG3) promoted binding by 63% of the macrophages, but fewer than 22% of these macrophages were able to phagocytose the parasites (Table IV).

Since some monoclonal antibodies were not able to mediate macrophage binding of trypanosomes *in vitro*, we analyzed whether addition of ineffective monoclonal products would interfere with the uptake that occurred in the presence of functional monoclonal antibodies. Also, since other laboratories (Hall & Esser, 1984; Ehrlich *et al*. 1982) have shown synergistic effects occurring when two monoclonal antibodies are mixed, we were interested in determining whether a similar phenomenon occurred in our system.

Resident peritoneal macrophages were incubated for 48 hours in Lab-Tek chambers. 20ul of the first monoclonal antibody and varying volumes (0-140ul) of the second monoclonal antibody were mixed in tubes. 0-140ul of normal mouse serum was added to keep total volumes constant. 150ul of these mixtures were added to an equal volume of WRATat-1 organisms at a concentration of 75 x 10^6 trypanosomes/ml. These slides were incubated for 30 min at 37°C, washed, stained, and the macrophages assayed for trypanosome-binding.

TABLE IV

The relative ability of monoclonal antibodies to mediate binding and phagocytosis of *trypanosomes*

% macrophages with trypanosomes that were:

Monoclonal Antibody	Bound only	Bound and/or internalized	Negative
IgM			
31.4D1.6.1	1	0	99
32.2A1.1	0	96	4
32.2A4.1	11	48	41
32.2A6.1	0	0	100
32.4G10	14	39	47
IgG1			
2.1B7.1	6	48	36
6.7H11	3	32	65
6.10A4.1	9	42	49
6.10F11.1	7	32	60
6.11B4.1	3	55	42
6.11D5.2	14	68	18
6.12E4	4	63	33
IgG2a			
16.1A5.3	36	49	15
16.1A8.1	9	53	38
IgG2b			
4.1G9.1	1	0	99
IgG3			
15.2H5.1	41	22	37
31.4G5.1	1	0	99
32.4G15.1	1	0	99
IgA			
6.11A9.0	5	10	85

Those monoclonal antibodies that had in previous assays exhibited a strong ability to promote binding, were not inhibited by the addition of monoclonal antibodies that had previously proven unable to mediate binding (Table V). In fact, the addition of increasing quantities of ineffective monoclonal antibodies frequently enhanced uptake compared to that which was obtained when the effective monoclonal antibodies were used alone. In the latter example, the antibodies by themselves were responsible for 79% and 34% binding respectively. When 140ul of 31.4G5.1 (a monoclonal antibody which was itself unable to mediate binding of parasites to macrophages) was added to the primary monoclonal antibody, uptake was increased to 93% and 64% (Table IV, lines 1 and 2).

Synergistic responses were also found with those monoclonal antibodies usually capable of only intermediate levels of binding. 20ul of 6.12E4 (d) in the absence of other monoclonal products promoted the binding of trypanosomes to 26% of macrophages (Table V, line 3). 6.7H11 (e) was a monoclonal product also able to mediate only low levels of binding (Table V, line 4). When these two monoclonal antibodies were combined, depending on the amount of secondary antibody added, 43%-54% of the macrophages were able to bind trypanosomes (Table V, lines 3 and 4).

Addition of as much as 7-fold the amount of nonfunctional antibodies to monoclonal products which were capable of promoting only low levels of binding did not inhibit the ability of the latter reagents to mediate binding of trypanosomes to macrophages (Table V, lines 5 and 6).

TABLE V

Macrophage-Trypanosome Binding Induced by Mixtures of Monoclonal Antibodies*

	First (#1) Mab Alone	Second (#2) Mab Alone	Monoclonal Antibody Mixtures: 20 ul #1 / 32 ul #2	Monoclonal Antibody Mixtures: 20 ul #1 / 140 ul #2
1.	79(a)**	0(b)	73	93
2.	34(c)	0(b)	30	64
3.	26(d)	26(e)	43	54
4.	26(e)	26(d)	51	43
5.	11(f)	14(g)	13	20
6.	14(g)	11(f)	26	15

a. 32.2A1.1 (IgM)
b. 31.4G5.1 (IgG3)
c. 6.11D5.2 (IgG1)
d. 6.12E4 (IgG1)
e. 6.7H11 (IgG1)
f. 4.1G9.1 (IgG2b)
g. 6.11A9.0 (IgA)

* 32-14ul of the second monoclonal antibody (Mab) was added to 20ul of the first Mab. 150ul of the mixture was added to monolayers of macrophages simultaneously with 150ul of WRATat-1 trypanosomes.

** Results represent % macrophages binding trypanosomes.

E) DETERMINATION OF THE IMMUNOGLOBULIN CLASS DEPENDENCE OF MACROPHAGE-TRYPANOSOME BINDING IN VITRO

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 starting material (hyperimmune serum) produced 83% binding of trypanosomes by macrophages. 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 VI). 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.

F) ANALYSIS OF THE COMPLEMENT COMPONENTS AND PATHWAYS REQUIRED TO ENHANCE BINDING OF T. RHODESIENSE TO MACROPHAGES

The role of complement in resistance to African trypanosomiasis remains controversial (Ferrante and Jenkins, 1978; Takayangai et al, 1974). In previous studies (Greenblatt et al, 1983) we found that in the presence of limiting amounts of specific antisera, complement enhanced the uptake and degradation of trypanosomes by macrophages. This phenomenon was examined in greater detail.

Anti-Wellcome antisera was heat-inactivated at 53^{o}C for 90 min and used at various concentrations depending on the titer of the lot of antiserum

TABLE VI

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% binding)		
IgM		
without complement	85	0
with complement	76	0
IgG2a		
with or without complement	0	0
IgG2b		
with or without complement	0	0

used. In the presence of heat-inactivated antiserum diluted 1:35, only 13% of the macrophages bound trypanosomes. Upon addition of 6% fresh rat, guinea pig, or dog complement these percentages increased to maximum uptakes of 95%, 91%, or 68% respectively (Table VII).

Serum from guinea pigs genetically deficient in C4 ($C4D^{gp}$, a gift from Dr. Robert O. Webster, NYS Kidney Disease Institute, Albany, NY) has no detectable C4 function (May et al., 1971) and therefore completely precludes activation of the complement cascade by the classical pathway (May et al, 1972). This serum was used to determine the requirement for C4 and the classical pathway to enhance binding of trypanosomes to macrophages in the presence of limiting dilutions of antiserum. The addition of $C4D^{gp}$ anti-trypanosome antibody resulted in lower levels of total binding as compared to binding that occurred in the presence of intact guinea pig sera (66% vs 91%). Interestingly, there was no decrease in the phagocytosis of bound trypanosomes. These findings indicate that the classical complement pathway is involved primarily in the enhancement of binding of trypanosomes to macrophages.

To determine the role of the C3 component, dog serum deficient in functional C3 (C3D dog, a gift from Dr.Jerry Winkelstein, Johns Hopkins University, Baltimore, MD) was tested. When the C3D dog serum was added to anti-trypanosome antiserum there was only a slight decrease in the total phagocytosis of trypanosomes by macrophages (59% vs. 68%). However, the absence of C3 resulted in a significant decrease in the uptake of bound trypanosomes (25% vs 51%). These findings indicate that activation of complement via the alternative pathway and the C3 component are involved in the enhancement of phagocytosis of bound trypanosomes.

TABLE VII

Effect of the addition of complement deficient or intact sera on the binding of trypanosomes to macrophages.*

Fresh serum	Macrophages with attached trypanosomes	Macrophages with attached & internalized trypanosomes	% Total binding	Ratio of internalized trypanosomes to total binding
None	ND	ND	13	
C^{rat}	22	73	95	.77
$C^{guinea\ pig}$	2	89	91	.98
$C4D^{guinea\ pig}$	4	61	66	.92
C^{dog}	17	51	68	.75
$C3D^{dog}$	34	25	59	.42

* 20ul of medium or serum intact or deficient in complement components was added to 145ul of anti-Wellcome antiserum diluted 1:35. 150ul of these mixtures were added to an equal volume of Wellcome trypanosomes at a concentration of $75x10^6$ parasites/ml and added to Lab-Tek chambers containing 72 hr cultured macrophages. Slides were incubated 30min at 37C, washed, and stained.

Since it was possible that the cold inhibited trypanosome penetration as well as macrophage phagocytic activity, an alternate method for inhibiting macrophage uptake was used. Iodoacetic acid (IAA) is known to inhibit phagocytosis of sheep red blood cells (SRBC) by macrophages (Walker and Demus, 1975). IAA (1.5×10^{-3}) was therefore added to macrophages in the presence of SRBC and antiserum to SRBC. After incubation for 30 min at 37°C, macrophage monolayers were washed well with RPMI 1640 to remove all traces of the inhibitor, and in the presence of antiserum, trypanosomes were added to the cultures. Cultures were incubated again for 30 min at 37°C, and slides prepared.

As can be seen in control samples (Table VIII, lines 1 and 2), IAA did not significantly effect binding of SRBC (31% vs 22% for IAA and medium-treated macrophages respectively). It did however effect internalization of opsonized sheep red blood cells. In the presence of IAA, 23.5% of the macrophages had internalized SRBC as compared to 49% of non-treated cells. The inhibition of binding caused by IAA was actually more complete than illustrated by these data since there were much fewer internalized SRBC in the IAA heated cells than in the non-treated (data not shown).

The effect of IAA was much more pronounced with the trypanosomes. When trypanosomes and anti-trypanosome antisera were added to washed macrophages previously exposed to IAA there was a 30-fold decrease in the numbers of macrophages that had internalized trypanosomes.

These data confirm that T. rhodesiense trypanosomes do not actively penetrate macrophages, but that they are only taken up by phagocytosis.

TABLE VIII

Effect of Iodoacetic acid (IAA) on uptake of opsonized sheep red blood cells and trypanosomes.

Treatment of Macrophages	Particle[a]	Attached	Internalized	No Binding
IAA	SRBC	31.0	23.5	45.5
None	SRBC	22.0	49.0	29.0
IAA	Trypanosomes	69.5	1.5	29.0
None	Trypanosomes	49.0	44.0	7.0

[a] All particles were opsonized with an appropriate antibody.

These results indicate that both the classical and alternative complement pathways are involved in the enhanced binding and uptake of trypanosomes to macrophages in the presence of small amounts of specific antibody.

G) CHARACTERIZATION OF MACROPHAGE FUNCTIONS & RECEPTORS INVOLVED IN THE IN VITRO SYSTEM

Some trypanosomes, such as T. cruzi are able to actively penetrate host cells. However, the mechanism of cellular uptake of T. rhodesiense is not known. We therefore investigated whether this organism actively penetrated cells, or whether its uptake was solely by cellular phagocytosis.

Macrophages were incubated with parasites at varying temperatures in order to dissociate binding of trypanosomes from the internalization or phagocytosis of trypanosomes. The ingestion of, as compared to the attachment of large particles by macrophages is highly temperature dependent. Macrophages bind and phagocytose well at 37°C, but at 4°C little internalization occurs (Silverstein, 1977). 150½l of rat anti-Wellcome trypanosome antibody at dilutions of 1:2, 1:4, or 1:8, was added to an equal volume of Wellcome trypanosomes at a concentration of 75 x 10^6/ml parasites and incubated for 30 min at 4°C or 37°C. 80% of all macrophages were capable of binding trypanosomes when the macrophages were incubated at 4°C, but only 19% of the macrophages internalized parasites (data not shown). In comparison, when cells were incubated at 37°C, 39-75% of the macrophages, depending on titer of mediating antibody, were capable of both binding and digesting trypanosomes. These findings suggest that in this assay, African trypanosomes do not actively penetrate murine macrophages, but instead are phagocytosed by the macrophages.

H) ANALYSIS OF UPTAKE BY MACROPHAGES FROM RESISTANT AND SUSCEPTIBLE MICE.

In published reports (Greenblatt, et al., 1980, 1984) we have demonstrated major differences in resistance among various mouse strains to Trypanosoma rhodesiense (EATRO 1886). C57BL/6 mice are most resistant, while BALB/c and C3H/HeJ mice are most susceptible to this organism. Since the cellular basis of these differences are not known, it was of interest to determine if macrophages from these strains differed in their ability to phagocytose trypanosomes. Resident macrophages were removed from non-infected C57BL/6, BALB/c, and C3H/HeJ mice and cultured for 48-72 hours before use. 0.15 ml of Wellcome trypanosomes at a concentration of 75 x 10^6/ml was added to an equal volume anti-Wellcome antibody diluted from 1:5-1:25. Trypanosomes were permitted to adhere for 30 min at 37^{o}C before suspensions were washed off.

As can be seen in Table IX, as the titer of anti-Wellcome antibody decreased, the percentages of macrophages taking up trypanosomes also decreased. However, there were no significant differences in uptake between the three mouse strains.

BALB/c and B6 mice were next infected with the EATRO 1886 strain of T. rhodesiense and sacrificed on 10d postinfection. Peritoneal macrophages were removed from these mice and from non-infected age-matched controls, plated for 72 hours and assayed as usual with Wellcome trypanosomes and anti-trypanosome antibody. As can be seen from Table X, there was progressively less uptake of trypanosomes by macrophages from normal BALB/c and B6 as antiserum was diluted from 1:20-1:35. BALB/c macrophages removed 10d post infection took up trypanosomes more actively than normal controls at the higher dilutions of antiserum. The uptake of trypanosomes by infected B6 macrophages was also greater than the controls, but only at the highest titer of antiserum (1:20).

TABLE IX

The binding of trypanosomes in the presence of various dilutions of antiserum by macrophages from three different mouse strains.

Dilution of anti-trypanosome antiserum	C57BL/6	BALB/c	C3H/HeJ
1:5	82∓7	75.5∓18	ND
1:10	95.5±2	87.5±7	ND
1:20	86.5±5	80.3±6	84.5±14
1:25	72.6±13	79.2±11	78.0±18

A mixture of 150ul of diluted anti-Wellcome antiserum was added to an equal volume of Wellcome trypanosomes to obtain final concentrations of antiserum of 1:5-1:25 and added to cultured macrophages obtained from 3 different strains of mice. After incubation at 37C for 30min, slides were treated as usual.

TABLE X

The binding of trypanosomes in the presence of various dilutions of antiserum by macrophages from non-infected and infected mice.

	Dilution of Anti-Trypanosome Antiserum			
PEC origin	1:20	1:25	1:30	1:35
Normal BALB/c	73.5±8	60±2	17.5±.5	6.5±3
d10 Infected BALB/C	86.5±.5	44.5±8	37.5±4*	38.5±11*
Normal B6	78.5±6	67±7	14.5±5	28±2
d10 Infected B6	30.5±4*	48±2	19±4	18±2*

* Statistically significant (Student's T test)

A mixture of 150ul of diluted anti-Wellcome antiserum was added to an equal volume of Wellcome trypanosomes to obtain final concentrations of antiserum of
1:20-1:35 and added to cultured macrophages obtained from non-infected and infected BALB/c or C57BL/6 (B6) mice.

These findings indicate that trypanosome infection results in an increase in the phagocytic activity of macrophages. However observed macrophage strain differences do not seem to be of sufficient magnitude to account for the differences in survival.

I) USE OF CONTINUOUS CELL LINES AS INDICATOR CELLS

1. Murine Lines

Since cell collection for the *in vitro* assay is time consuming, and since the use of cells from living animals is a potential source of uncontrolled variability, it was important to determine if cell lines could be used as indicator cells in this assay. Five murine macrophage cell lines were therefore tested for their ability to bind and phagocytose trypanosomes *in vitro*. One line, J774.2, bound organisms very well (90%), but phagocytosed poorly (1%) (Table XI). J774.16, a functional variant of J774.2, also bound organisms well (92%+), and was slightly more phagocytic. J774.3.4 a Fc-receptor deficient mutant, bound organisms less well (51%+) than the parent cell. CTRM1, an adenyl cyclase deficient mutant, bound cells relatively poorly (33%+), but surprisingly appeared to be the most phagocytic cell line. P388D1, another murine macrophage cell line, did not function as well in this assay as did the J774 set of cells, with only 33% of cells binding, and 17% phagocytosing organisms. Thus no murine macrophage cell line was found that functioned as well as normal macrophages. However, screening of additional lines may uncover one with greater functional activity. Furthermore, the presence of functional deficiencies between cell lines with defined biochemical abnormalities, may help to elucidite the relevant mechanisms by which macrophages bind trypanosomes.

TABLE XI

The ability of murine macrophage cell lines to bind trypanosomes in the presence of trypanosome specific antibody

Cell Line	Origin	Attached Properties	% Binding: Attached and Only	% Binding: No Internalized	% Binding: Binding
J774.2	murine reticulum cell sarcoma (J774)	-secretes lysozyme -phagocytizes latex and opsonized particles -secretes plasminogen activator -possesses Fc-receptors for IgG2a, IgG2b, IgG1, and IgG3	89	1	10
J774.16	variant of J774	-can be induced to oxidize glucose via the hexose monophosphate shunt -produces H_2O_2 and O_2	80	12	8
J774.3.4	variant of J774.2	-decreased Fc receptors -decreased phagocytosis of IgG2a & IgG2b opsonized particles -augmented IgG2a particle uptake in presence of cAMP	51	0	49
CTRM1	variant of J774.2	-adenyl cyclase deficient	10	23	67
P388D1	variant of murine macrophage-like cell from P388 leukemia cells	-can bind antigen antibody complexes of mouse IgG2a and rabbit IgG -produces interleukin-1	33	1	66

2. Human cell lines

We also screened a number of newly developed human cell lines for their ability to bind trypanosomes in the presence of anti-trypanosome antiserum.

Four human macrophage cell lines (BB, CG, CS and DM) were derived by Dr. Betty Diamond of Albert Einstein College of Medicine. All 4 cell lines produce lysozyme and collegenase, and are esterase positive. None of the cell lines are capable of producing Interleukin-1 (IL-1). Approximately 80% of the cells in each clone have complement receptors and all have Fc receptors as measured by uptake of opsonized erythrocytes.

In our hands, adherence of these cells to Lab-Tek chambers was poor. However, of those cells that did adhere, 49%-72% were able to bind Wellcome trypanosomes in the presence of specific antibody (Table XII).

DISCUSSION

The findings presented in this report demonstrate that the *in vitro* macrophage assay is a useful model system for analyzing the interaction between macrophages, trypanosomes and antibodies. One major advantage of this assay is for the screening of antibodies for their potential ability to neutralize trypanosomes *in vivo*. Of the 32 monoclonal antibodies tested, no antibody that was negative *in vitro*, had *in vivo* activity. However, the assay does generate some false positives and is therefore only useful as a first order screening method. Thus, six monoclonal antibodies that mediated binding to macrophages *in vitro*, were unable to neutralize *in vivo*.

Four of these bound exclusively to the flagellar pocket of the trypanosome, and this binding to the flagellar pocket could explain the discrepancy between the *in vivo* and *in vitro* results. The pocket of the parasite has traditionally been considered as a region through which much movement occurs. Also, antisera directed against *T. rhodesiense* caps

TABLE XII

The ability of human cell lines to bind trypanosomes in the presence of 1:20 anti-Wellcome antiserum

Cell Line	Percent Binding Trypanosomes
BB	49
CS	52
DM	72

150 ul of 1:10 titer of anti-Wellcome antiserum was added to an equal volume of Wellcome trypanosomes and added to monolayers of the human cell lines.

to an area about the flagellar pocket and is sometimes internalized (Gardiner et al, 1983). It is therefore possible that antibodies bound to the flagellar pocket might be rapidly internalized. By the time the parasite had been introduced into the host the antibody would no longer exist on the surface and the organism would no longer be opsonized. This lack of opsonization would prevent removal of the parasite.

The discrepancy with monoclonal antibodies 6.7H11 and 15.2H5.1 which exhibit neither fluorescence or neutralization, but were able to promote binding of trypansomes to macrophages is harder to explain. (Table III). These conflicting results may be due to differing lots of monoclonal antibodies utilized in the two separate assays. In order to reconcile this discrepancy, these experiments will have to be repeated using aliquots of the same lot of monoclonal antibodies.

The assay is also useful for characterizing the type of interaction between macrophages and trypanosomes that is mediated by individual antibodies. Thus, while binding promoted by most antibodies is associated with parasite internalization, some antibodies seem to preferentially mediate binding only (Table IV). It is noteworthy that one of the antibodies that has this property (15.2H5.1), binds to the flagellar pocket, and does not neutralize trypanosomes in vivo.

Using this assay, it was also possible to demonstrate that some antibodies that were themselves unable to mediate binding, could enhance the activity of other antibodies (Table V). It is interesting how a monoclonal antibody, which by itself is unable to mediate binding or uptake of parasites by macrophages, is nevertheless able to enhance the binding potential of a second monoclonal antibody. Hall and Esser (1984) have also found in their system that a monoclonal antibody which was not able to bind to live

organisms, was nonetheless able to increase the binding of another monoclonal. However, in contrast to our findings, they were also able to demonstrate that some monoclonal antibodies were able to block others.

When the activities of natural antibodies (as opposed to monoclonal antibodies) were examined *in vitro*, it was found that only the IgM antibodies could mediate trypanosome binding by macrophages (Table VI). 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.

These data differ from our findings with the monoclonal antibodies. 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.

The assay also allows the analysis of the importance of serum components other than antibody in the uptake of trypanosomes by macrophages. It was found that serum complement is an important cofactor in this system, and that complement activation by both the classical and alternative pathways was involved (Table VII). Classical pathway activation was most important in the enhancement of binding of trypanosomes while intact C3 was important for internalization of already bound trypanosomes. These findings may be

important in helping to elucidate the relative role of non-specific host defense mechanisms involved in combating trypanosome infections.

The importance of macrophage function in determining murine host resistance to T. rhodesiense infection was also determined using this assay system. C57Bl/6 mice are resistant to this infection, while BALB/c mice are susceptible. No difference in the endogenous ability of macrophages of these two strains to bind trypanosomes was found (Table IX), suggesting that genetically determined differences in resistance are not expressed in the resting function of these cells. However, the response of macrophages from these two strains to infection was significantly different (Table X). Whether this reflects a primary difference in macrophage function, or is a secondary manifestation of the infection remains to be established.

A variety of murine and human macrophage cell lines were also screened for their ability to bind and internalize trypanosomes in vitro. No lines were found to behave identically to normal macrophages. Thus, while these lines may be useful in examining the role of specific macrophage properties in this assay, those tested so far cannot be substituted for normal cells.

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