Immunology 1980 **40** 317

Fc receptors of rat peritoneal macrophages: immunoglobulin class specificity and sensitivity to drugs affecting the microfilament or microtubule system

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Accepted for publication 17 January 1980

Summary. Macrophage-cytophilic antibody activity of various immunoglobulin classes and subclasses was tested in two different rosetting systems. Cytophilic antibody activity of IgM, IgG2a and IgG1 was verified in the SRBC system, while IgM, IgG2a and IgG2c were found to be active in the trypanosome system. Sensitivity to cytochalasin B treatment of SRBC rosette formation was dependent on the class of antibody and decreased in the following order: IgM > IgG1 > IgG2a. Trypanosome rosette formation was prevented by the same drug regardless the type of antibody. Vinblastin caused an enhancement of rosette formation in the SRBC system in low concentration, except when the antibody belonged to subclass IgG1. The enhancing effect was less pronounced in the trypanosome system.

INTRODUCTION

Adherence of particulate antigens to macrophages (rosette formation) in the presence of antibodies has been widely used to evaluate the capacity of various immunoglobulin classes and subclasses to mediate interaction between antigens and macrophages. Besides the conventional SRBC rosetting we also used a blood parasite, *Trypanosoma equiperdum* as antigen. In this system, attachment of living and moving organisms to the macrophages was observed as in the study of Takayanagi, Nakatake & Enriquez (1974). Comparison of the SRBC with the trypanosome rosette tests revealed differences in both the antibody and the cellular requirements of antigen fixation to macrophages.

The role of cytoskeletal structure in the activity of macrophage receptors have been established (Atkinson & Parker, 1977; Atkinson, Michael, Chaplin & Parker, 1977; Passwell, Schneeberger & Merler, 1978). We have found rosette formation displaying different sensitivities to microfilament damage (cytochalasin B) and to microtubule disruption (vinblastin) depending on the class of antibody involved.

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0019-2805/80/0700-0317$02.00

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MATERIALS AND METHODS

Animals

Inbred (R/LATI 78, cc $H\text{-}1^{w}$) Wistar male rats weighing 200–250 g were used.

Trypanosoma equiperdum

The strain was maintained in outbred CFLP mice by passage every 72 h.

Trypanosome antigens

Rats were exsanguinated by aortic puncture 48 h after infection by trypanosomes. The citrated blood was centrifuged and the trypanosome ring above the erythrocyte button was aspirated. The trypanosomes were washed twice in Hanks's balanced buffer solution (HBBS) and suspended in HBBS containing 1% glucose. The suspension contained 8×10^7 parasites/ml. Formalin was added to a 0·1% final concentration. This antigen preparation will be designated as T_f.

Soluble trypanosome antigen was obtained by suspending 8×10^7 trypanosomes per ml of water, freezing and thawing repeatedly and sonication. This antigen will be designated as T_s.

Immunization

Both antigens were mixed with Freund's complete adjuvant (1:1, v:v) and each animal was injected with 0·4 ml of the emulsion in the hind footpads subcutaneously. After 14 days, another 0·2 ml antigen was given without adjuvant, and on day 7 following the second injection the animals were exsanguinated. The sera were pooled and stored at $-20°$. Antiserum against sheep red blood cells (SRBC) was prepared similar way using a 30% suspension of SRBC.

Isolation of immunoglobulin (sub) classes

Immune sera were extensively dialysed against 0·5% boric acid to precipitate euglobulins. The precipitate was resolved into an IgM-containing macroglobulin fraction and another one containing mainly IgG2c with some IgG2a and IgG2b. IgG2c was further purified by affinity chromatography on Protein-A Sepharose (Pharmacia). The crude fraction was applied in 0·1 M phosphate buffer pH 7·0, and the column was subsequently washed by the same buffer, then 0·1 M phosphate buffer pH 6·0. IgG2c was eluted by 2 M $MgCl_2$.

The supernate was dialysed against 0·005 M sodium phosphate buffer pH 8·0 and applied on a DEAE cellulose (DE 52, Whatman) column in the same buffer. The fraction recovered in the starting buffer was pure IgG2a. Stepwise elution by buffers of increasing molarities yielded IgG2a- plus IgG2b-containing fractions (0·015 M), IgG2a- plus IgG2b- and IgG1-containing ones (0·05 M), while an IgG1-containing fraction was eluted by 0·3 M buffer. Pure IgG2b was obtained by Protein-A Sepharose chromatography of the IgG fraction eluted from the DEAE cellulose by 0·015 M phosphate buffer: IgG2a being recovered in 0·1 M phosphate buffer pH 6·0.

Fractions were assayed by immunoelectrophoresis using polyvalent serum against rat serum proteins as well as by immunodiffusion using monovalent antisera against rat immunoglobulin classes or subclasses, respectively. Monospecific antisera were prepared as described previously (Bazin, Beckers, Querinjean, 1974). Monoclonal immunoglobulins representing single (sub)classes were isolated from sera of Lou rats bearing immunocytoma (Bazin *et al.*, 1974).

Macrophage Fc-receptor activity

This was tested adapting the method of Mazzoli & Barrera (1973). The production of peritoneal macrophages was provoked by 10 ml of 0·1% sterile glycogen solution. Five days later the peritoneal cavity was washed with HBBS containing 5 i.u. heparin/ml (Richter, Budapest). The cells were cultured in petri dishes (Nunclon, N-1420, 40 × 12 mm) in an atmosphere of 5% CO_2, 95% air at 37°. After 2 h, the non-adherent cells were removed by washing the monolayer twice with HBBS. The adherent cells were resuspended by the use of a 10^{-4} M EDTA solution and a rubber policeman. The cells were washed three times and resuspended in medium RPMI 1640 (GIBCO) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The number of viable cells was determined by trypan blue dye exclusion in a Buerker chamber.

Two hundred microlitres of the 2×10^6 cell/ml suspension was put into the Micro Test Plate (Nunclon N-1480). Thirty minutes later the non-adherent cells were removed by vigorous washing, 98% of the adherent cells were mononuclear ones with Fc receptors. For testing cytophilic antibody activity, 200 μl of the appropriate serum dilution or isolated antibody solution was applied onto the monolayers. Unbound proteins were removed by thorough washing after 60 min and a 10% SRBC suspension or living trypanosomes, 5×10^6 organisms/ml, respectively was applied on the monolayers. The percentage of rosette forming cells was determined on the native preparation.

In inhibition tests, the appropriate immunoglobulin preparations were added simultaneously with the cytophilic antibodies. Cytochalasin B (Calbiochem) or vinblastin (Richter, Budapest) were applied to the monolayer before the addition of antibodies. After 60 min incubation the drugs were removed by extensive washing.

RESULTS

Rosette formation of trypanosomes around macrophages

Adherence of trypanosomes to macrophages was

Figure 1. Trypanosome rosettes around rat peritoneal macrophages, from a microcinematographic film.

observed only in the presence of specific antibodies; in presence of normal rat serum trypanosomes could be washed away from the monolayer. Living parasites adhered to macrophages by their oral poles and they performed a restless, quick vibrating movement while remaining captured. Two different forms of trypanosome clustering were observed, a bunch form and a sun-disc like form (Fig. 1). In the former, trypanosomes are fixed to the cell at points in close proximity, mostly on processes of the macrophages; in the latter the moving parasites surround the macrophages like erythrocytes in an SRBC rosette. In Fig. 2, the relative number of rosette-forming macrophages is plotted against the dilution of the antiserum. Both anti-trypanosome sera exhibited a titre comparable with that of an anti-SRBC serum prepared by the usual technique.

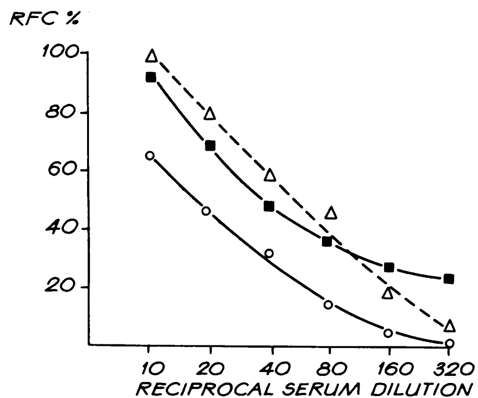

Figure 2. Dependence of rosette-forming cell frequency on antiserum concentration: ■, anti-SRBC serum; △, anti-T_s serum; ○, anti-T_f serum.

Assignment of macrophage cytophilic antibody activity to immunoglobulin classes and subclasses

Sera containing cytophilic antibody activity were separated into fractions containing pure or partially purified immunoglobulin classes or subclasses. MCA activities of fractions derived from the three different sera are demonstrated in Table 1. MCA activity was tentatively assigned to IgM, IgG2a and IgG1 in the case of the anti-SRBC serum, to IgG2a and IgG1 in the case of the anti-T_f serum and to IgM, IgG2c and IgG1 in the case of the anti-T_s serum. The tentative assignment was checked by testing the capacity of purified monoclonal immunoglobulin derived from immunocytoma-carrying rats representing the IgM class and the IgG subclasses to inhibit the MCA activity of the various antibody fractions (Table 2). The inhibition tests verified the tentative assignment with the exception of the anti-T_f serum, in which the only immunoglobulin capable of mediating trypanosome rosette formation appeared to be IgG2a. Cross-inhibition was found in several instances. IgG2a could inhibit the anti-SRBC IgG1-type antibodies and the IgG2c-type antibodies from the anti-T_s serum. IgG2c was found to inhibit anti-SRBC IgG2a. No significant inhibition of IgM type antibodies by IgG subclasses could be detected.

Cytochlalasin B and vinblastin sensitivity of rosette formation mediated by antibodies of different immunoglobulin class

We have compared the effect of cytochalasin B and vinblastin on the trypanosome and SRBC rosette formation mediated by isolated IgM, IgG1, IgG2a or IgG2c antibodies (Table 3). The SRBC rosettes with IgM antibodies are highly sensitive to cytochalasin B while those formed with IgG1 type antibodies are exceedingly sensitive to vinblastin. SRBC rosette formation with IgG2a antibodies was found to be relatively resistant to both drugs. Trypanosome rosette formation was found to be more sensitive to cytochalasin B than SRBC rosette formation, regardless of the class of antibody, while the vinblastin sensitivity of trypanosome rosette formation was similar to that of SRBC rosette formation. An increase in SRBC rosette formation was observed by vinblastin at 10^{-7} M apart from the IgG1 mediated rosettes. A similar enhancing

Table 1. Frequency of RFC in macrophage monolayers incubated with isolated antibody fractions

Antigen	Antibody fractions: Protocol No.	Antibody fractions: (Sub)classes present	Protein concentration (µg/ml): 250	Protein concentration (µg/ml): 500
SRBC	25/I	IgM	35	68
	25/II	IgG2c, IgG2a	0	5
	34/III	IgG2c	0	0
	24/I	IgG2a	40	95
	24/II	IgG2a, IgG2b	55	65
	24/III	IgG1, IgG2a, IgG2b	68	98
	24/IV	IgG1	75	81
T_f	522/I	IgM	0	0
	522/II	IgG2c, IgG2a	0	5
	524/III	IgG2a, IgG1	12	38
	524/IV	IgG2a	51	72
T_s	27/I	IgM	25	46
	27/II	IgG2c, IgG2a	55	73
	31/III	IgG2c	30	56
	26/I	IgG2a	8	14
	26/II	IgG2b, IgG2a	18	25
	36/II	IgG2b	0	0
	26/III	IgG1, IgG2a, IgG2b	0	0
	26/IV	IgG1	0	0

Table 2. Macrophage-cytophilic antibody property of rat immunoglobulin (sub)classes as studied by inhibition of rosette formation by purified monoclonal proteins

Antigen	Antibody fraction 250 μg/ml	Inhibitor 1 mg/ml		RFC%
		—		28
SRBC	25/I, IgM	IR	IgG1	35
		IR 202	IgM	0
		IR 418	IgG2a	48
		—		45
	24/1, IgG2a	IR 202	IgM	56
		IR 418	IgG2a	0
		IR 64	IgG2c	28
		IR	IgG1	45
		—		55
	24/II	IR 418	IgG2a	0
	IgG2a, IgG2b	IR	IgG2b	49
		—		68
	24/III	IR 418	IgG2a	0
	IgG2a, IgG2b, IgG1	IR	IgG2b	71
		IR	IgG1	18
		—		70
	24/IV IgG1	IR 202	IgM	72
		IR 418	IgG2a	49
		IR	IgG1	15
		—		12
T_f	524/III	IR 202	IgM	25
	IgG2a, IgG1	IR 418	IgG2a	0
		IR	IgG1	18
		—		64
	524/IV IgG2a	IR 202	IgM	25
		IR 418	IgG2a	0
		IR	IgG1	18
		—		20
T_s	27/I, IgM	IR 202	IgM	5
		IR 418	IgG2a	28
		IR 64	IgG2c	20
		IR	IgG1	32
		—		46
	27/II	IR 202	IgM	48
	IgG2c, IgG2a	IR 418	IgG2a	0
		IR 64	IgG2c	21
		IR	IgG1	51
		—		38
	31/III IgG2c	IR 202	IgM	30
		IR 418	IgG2a	16
		IR 64	IgG1c	0
		IR	IgG1	25
		—		15
	26/II	IR 418	IgG2a	0
	IgG2a, IgG2b	IR	IgG2b	15
		IR 64	IgG2c	24
		IR	IgG1	25

effect on trypanosome rosette formation was found only in the case of the IgG2c-type antibody.

DISCUSSION

The three different antisera involved in this study were found to differ in the immunoglobulin isotype specificity of their macrophage-cytophilic antibody (MCA). In the anti-SRBC serum, MCA activity was detected in IgM, IgG2a and IgG1 but not in IgG2b or IgG2c. The difference between the two anti-trypanosome sera was remarkable: in the anti-T_f serum only IgG2a was found to carry this activity while in the anti-T_s serum IgM and IgG2c was also active. No MCA activity was detected in IgG1 of either anti-trypanosome serum.

Interaction of various IgG subclasses with macrophages has been demonstrated by many authors. Regarding the binding of IgM, controversial results have been published. In our systems, the capacity of IgM to mediate rosette formation around macrophages has been verified in concordance with Tizard's (1969) report of cytophilic activity of mouse macroglobulin antibodies to SRBC. Interaction between macrophages and IgM was also demonstrated by Lay & Nussenzweig (1969) who described Ca^{2+}-dependent rosetting of IgM antibody-sensitized SRBC around macrophages and by Walker (1977) who presented evidence for IgM mediated phagocytosis by macrophages.

The receptors for the various isotypes does not seem to be identical. No cross-inhibition was observed between IgM and any IgG subclass. A certain overlapping between IgG2a- and IgG1-binding receptors can be deduced from inhibition data in the SRBC system and between IgG2a and IgG2c as suggested by inhibition data in the trypanosome system. Heterogeneity of macrophage Fc receptors with respect to isotype specificity has also been found by Unkeless & Eisen (1975), Heusser, Andersson & Grey (1977) and Diamond, Bloom & Scharff (1978).

Rosette formation mediated by different isotypes exhibited varying sensitivity to cytochalasin B and vinblastin treatment of the cells. Differences were observed among isotypes in the same antigen–antibody system and also between the sensitivities of SRBC and trypanosome rosette formation when mediated by IgG2 antibodies.

According to a recent report by Passwell *et al.* (1978), the immunoglobulin binding capacity of

Table 3. Effect of cytochalsin B or vinblastin pre-treament on rosette formation of macrophages mediated by different (sub)classes of antibodies

Antigen	Antibody	No drug	Cytochalasin B (μg/ml) 100	10	1	Vinblastin (M) 10^{-6}	5×10^{-7}	10^{-7}
SRBC	Serum, 1:40	35	0	15	27	15	36	68
	IgM	33	0	0	0	45	42	57
	IgG2a	45	18	16	25	31	35	85
	IgG1	67	0	12	10	0	0	0
T_f	Serum, 1:40	36	0	0	0	5	24	46
	IgG2a	42	0	0	0	0	31	45
T_s	Serum, 1:40	44	0	0	0	0	35	38
	IgM	28	0	0	0	25	18	34
	IgG2c	32	0	0	0	0	10	55

monocytes is much less influenced by cytochalasin B than their ability to form rosettes with antibody-sensitized erythrocytes. In the same study, rosette formation was reported to require metabolic activity of the cell. According to this concept, rosette formation requires a certain clustering of receptor-bound antibodies to form a sufficient number of interaction with the multivalent corpuscular antigen. The importance of receptor mobility in binding large IgG aggregates by macrophages was also deduced from kinetic studies by Knutson, Kijlstra & van Es (1977). The dependence of stable antigen binding on the antibody clustering and thus on receptor motility may vary according to the receptor–antibody system. In a recent report, Diamond *et al.* (1978) described the cytochalasin B sensitivity of rosette formation of mouse IgG2a-antibody-coated SRBC and the resistance to the same drug in the case of mouse IgG2b-type antibodies. The differing sensitivity of rosettes with the two different antigens mediated by rat IgG2a found in the present study may reflect a stronger dependence of antigen adherence on the appropriate clustering of binding sites, since in this case, the antibody–receptor interactions and the antigen–antibody bonds have to counteract the movement of the parasite. The properties of the antigen must be taken into account when evaluating the possible role of a given antibody in mediating interactions between macrophages and a particulate antigen.

Our data about the effect of vinblastin, known to produce a disruption of the microtubule system, support the results of Passwell *et al.* (1978). In low concentration, an enhancement of the rosette-forming cell frequency was observed in the SRBC system. The extreme sensitivity of IgG1-mediated SRBC adherence suggests that this latter type of antibody can fix a particulate antigen to macrophages only in the presence of an intact microtubule system. One could certainly raise the question whether the binding of the antibody itself is not influenced by vinblastin in that particular case. Experiments are in progress to find a definite answer.

ACKNOWLEDGMENTS

The kind co-operation of Dr L. Fridvalszky (Budapest) in microcinematography of trypanosome-macrophage interactions is gratefully acknowledged. The authors are also indebted to Ms Kinga Ambrus for her expert technical assistance.

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