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Rheumatoid factors in mice: plaque assay for homophile and heterophile rheumatoid factors

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Summary. It was found that a high proportion of IgM-producing cells secrete homophile or heterophile rheumatoid factors (RF). Injection of lipopolysaccharide (LPS) into CBA mice resulted in a sharp increase in total IgM and in RF-producing cells and in some cases resulted in an increase in RF-producing cells as a proportion of total IgM. RF plaques are indirect IgM in the sense that they require development by IgG2 (anti-IgM) antibody for visualization in a standard haemolytic plaque assay. The RF response to LPS is both dose- and time-dependent.

INTRODUCTION

Lipopolysaccharides (LPS) derived from Gram-negative bacteria have long been known as non-specific activators of immune responsiveness (AIr). This has been demonstrated both after the injection of intact animals (Primi, Hämmarstrom, Smith & Möller, 1977a), and after the addition of LPS to *in vitro* cultures of lymphocytes (Coutinho, Gronovicz, Bullock & Möller, 1975; Möller 1975; Primi, Smith, Hämmarstrom & Möller, 1977b). In all the experiments mentioned above, injection or addition of LPS resulted in an increase in antibody or cells secreting antibody to determinants (DNP-, NIP-. SRBC, etc.) not present on the endotoxin (LPS) molecule itself. Similar 'non-specific responses' have been reported after the injection of control groups of mice with adjuvant materials, for example *Bordetella pertussis* organisms (Bp), without the admixture of 'antigen' (Dresser, Wortis & Anderson, 1970). More recently in preliminary communications from two groups, it has been pointed out that LPS activates an increase in overall IgM production, a significant proportion of which is accounted for as being specific for xenogeneic IgG (Dresser & Popham, 1976), for cryptic (auto) antigens revealed by bromelain treatment of both self and not-self erythrocytes (Steele & Cunningham, 1978) and for syngeneic IgG (Dresser, 1978b). This latter IgM response against self IgG, designated as a homophile rheumatoid factor response, accounts for between 25% and 85% of the total number of IgM-producing cells enumerated by a reverse plaque assay modified from the original methodology of Molinaro & Dray (1974).

In this paper, the salient features of the methodology used for visualization of RF plaques is described, together with a description of some experiments which may help to define basic variables of the RF response to non-specific AIr.

MATERIALS AMD METHODS

Animals

CBA/Ca (syn, CBA/H) NIMR male mice aged between 6 and 12 months were used in the experiments described in this paper. The mice were bred in specific-

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pathogen free (SPF) conditions and maintained in a minimal disease (MD) unit during the course of experimentation.

Plaque assay

A standard version of the haemolytic (Jerne) plaque assay using microscope slides was employed (Dresser, 1978a). Spleen cells were prepared in a standard manner and were suspended in Hanks's balanced salt solution containing 0·5% w/v gelatin (Difco, Bacto) (HG). Tubes containing aliquots of gel medium (0·2 ml) were maintained at 35° in a water bath, developing serum, target (indicator) erythrocytes and spleen cells were added to each tube and the mixture spread quickly onto a warmed microscope slide (35°), to form a thin even layer. Rapid setting of the low gelling temperature agarose (LGT) was achieved by transferring the slide to a cooled level plate. After setting the slide was inverted onto a plaquing tray for incubation at 37°. Complement addition, further incubation, fixation in glutaraldehyde followed by washing and drying the slides and enumeration of the plaques were all as described previously (Dresser, 1978a). In a limited number of experiments slides were reduced (DTT) and alkylated after incubation and immediately prior to complement addition, using the methodology of Plotz, Talal & Asofsky (1968).

The quality of the rheumatoid factor (RF) and the reverse plaques was improved by an increase in the pre-complement incubation time from 2 to 3 h, and by the use of LGT agarose (Marine Colloids Inc.) at a final concentration of 0·75% w/v in Eagle's tissue culture medium.

Preparation of target cells

Erythrocytes (RBC) for use as target (indicator) cells in the plaque assay were coated with IgG from various sources, using the chromic chloride method. RBC stored for 10–30 days at 4° in the form of whole blood in Alsever's solution were washed four times in plain saline (0·15 M NaCl). It is essential that prior to completion of coating all RBC suspensions are kept free of phosphate ions. To 1 vol of 50% RBC (packed cell volume) was added 10 vols of a saline solution of protein (0·25–0·5 mg/ml for MIG, 1–2 mg/ml for most other IgG preparations). The temperature was lowered to 0° in an ice bath and a small magnetic flea was added to the mixture. With stirring, 10 vol of 0·05 mM $CrCl_3$ freshly dissolved in saline (0°) was added dropwise. After stirring for a further minute the mixture was incubated at 37° for 10–20 min with gentle stirring, followed by the addition of HG (the phosphate stopped the coupling reaction) followed by two or three washes in HG. Coupled cells stored in HG at 0–4° were stable for several days.

Commercial Cohn ethanol fraction II preparations of several mammalian immunoglobulins have been found to be satisfactory: these are designated as IG, BIG is for example bovine immunoglobulin Fraction II. BIG as purchased (from Armour) had been further purified by passage through a column of DEAE cellulose (DE 23, Whatman) in 0·01 M Tris–HCl buffer, pH 8·1, containing 0·02 M NaCl.

MIG was prepared from mouse serum in three stages. Globulins precipitated in half-saturated ammonium sulphate (4°) were dialysed against 0·01 M Tris, pH 8·1, buffer containing 0·02 M NaCl, and passed through a column of DEAE-cellulose (DE 52) in the same buffer. The eluted material was then precipitated in 25% v.v. ethanol in 0·075 sodium acetate buffer at pH 6·7 (modified from Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946) by the following procedure. One volume of aqueous protein solution was placed in an ice-salt freezing mixture and stirred until ice crystals just start to form (between -1 and -2°). At this point, an equal volume of 50% ethanol in 0·15 M sodium acetate pH 6·7, at a temperature of -10 to -20°, was added dropwise at a rate which resulted in a steady drop in temperature of the mixture to between -5 and -7°. After centrifugation at -6° the supernatant was discarded and the precipitate dissolved in saline at 0°. On the advice of Dr Monique Bermann (University of California at Irvine, California) MIG preparations have recently received an additional mild acid treatment with an improvement in the quality of MIG (homophile RF) plaques. A MIG solution was adjusted to pH 4·0–4·1 in an approximately 0·1 M acetate buffer for incubation at 37° for 1 h. The pH was returned to 7 by the addition of dilute NaOH and the protein then dialysed against several changes of saline (0·15 M NaCl).

The goat IgG anti-(mouse)-μ was purified from the serum of a single hyperimmunized goat (GO 9). This animal was injected at 6–12 month intervals alternately with 4–10 mg MOPC 104E ($\mu\lambda_1$) and TEPC 183 ($\mu\kappa$) proteins in Freund's complete adjuvant. After two initial injections made intramuscularly, all subsequent injections were subcutaneous. The method used for preparing IgG from serum for use in the reverse plaque assay, was identical to that used for MIG, except that it was neither ethanol-fractioned nor acid-treated, nor was it necessary to use affinity

methods to prepare 'pure' anti-μ antibody. It was shown by passage of the IgG preparation of this antiserum down a μ-specific immuno-absorbent column (Sepharose 4B-TEPC 183), that 30–40% of the IgG had anti-μ activity. The goat anti-(mouse)-Fab was also from a single animal (GO 85) given multiple immunization injections and the IgG was made in a similar manner to the method outlined above.

Guinea-pig (IgG1 and IgG2) anti-(mouse)-μ globulins were prepared from the serum of animals immunized with myeloma protein (TEPC 183) in FCA followed by a boost of TEPC 183 protein in Freund's incomplete adjuvant (FIA). After precipitation in half-saturated ammonium sulphate, the globulins were dialysed against 0·01 M Tris–HCl, pH 8·1, and then adsorbed to DEAE cellulose (DE 52) in the same buffer. Different immunoglobulin isotypes were then eluted sequentially by passing a 0–0·1 M gradient of NaCl in the same buffer through the column. A protein (OD_{280}) peak eluted at 0·02–0·03 M NaCl was found on immunoelectrophoretic analysis to be IgG2 and a peak at 0·055–0·075 M was IgG1. These materials were used as indirect-μ developing reagents in the experiments illustrated in Fig. 2.

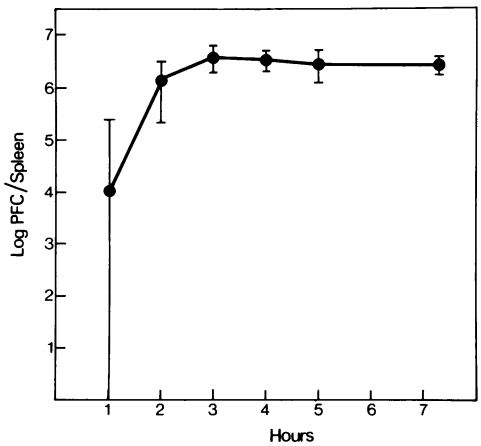

Figure 1. The effect of (pre-complement) incubation time on the number of detectable homophile RF (MIG) plaques. In addition to the apparent quantitative difference, plaque quality was markedly improved with increasing time of incubation up to 3 h. Each point and bar represents the mean and standard deviation of counts from four slides. Pool of spleen cells taken from four mice injected with LPS 4 days previously.

RESULTS

Plaque assay

Although the basic haemolytic (Jerne) plaque assay procedure was used, two minor modifications of the standard 'slide methodology' were found to improve RF and reverse plaque quality. LGT agarose gives better plaques on a purely subjective assessment, also a lengthened incubation time of 3 h before complement addition was shown to give slightly higher numbers than the conventional 1½–2 h (Fig. 1). As judged subjectively, plaques at 3 h were clearer than after 2 h of incubation.

μ-development

IgM reverse and RF plaques required development with a rabbit anti-(mouse)-IgM serum. In Table 1, it can be seen that addition of the developing serum at the start of incubation is more effective and economical than late addition; except in certain experiments not discussed in this paper, developing sera are always added directly to the gel medium at the start of incubation.

Data are presented in Table 2 to show that the developing potential of a rabbit anti-(mouse)-μ serum is removed on absorption with an IgM-specific immunoadsorbent, and that while potentiating the development of heterophile RF plaques the same concentration of anti-μ serum reduces the number of uncoated SRBC-specific plaques by about two thirds. An anti-κ chain serum with no demonstrable anti-μ activity developed RF plaques suggesting that any part of the IgM molecule can act as a target for the developing anti-globulin reagent.

The IgM nature of heterophile (BIG) RF plaques was confirmed in several experiments by their sensitivity to reduction by dithiothreitol (DTT) using the method of Plotz *et al.* (1968). Since the concentrations of DTT used were shown not to affect mouse IgG plaques against sheep RBC, it is assumed that rabbit IgG anti-(mouse)-μ is not affected either and consequently that the sensitivity of RF plaques is due to a direct effect of the DTT on the RF and not indirectly through destruction of the anti-μ developing antibody.

Figure 2 illustrates two points, first that anti-μ developing sera have well defined concentration optima, second that guinea-pig antibody of IgG2 isotype [and anti-(mouse)-μ specificity] develops RF plaques, while at the same concentration inhibiting SRBC plaques, whereas the IgG1 isotype inhibits both RF and other

Table 1. Greatest efficiency of plaque development when antiserum is added at the start of incubation

Developing serum		PFC/10^6 spleen cells × 10^3	
Relative concentration	Time (h)*	Total IgM	Anti-MIG†
×1	0	45·1	8·1
×2	2	30·4	0·4
×4	2	49·9	5·2
×1	3	0·6	0·4
×2	3	14·8	0·5
×4	3	25·3	2·3

* Hanks's gelatin (HG) flooded between gel layer on inverted slide and plaque tray at start of experiment. Time (h) indicates where HG replaced by HG containing rabbit anti-(mouse)-μ developing serum at 1, 2 or 4 times the optimum for plaque development in a conventional assay where the developing serum is added to the gel and HG is not flooded between slide and tray until the time of complement addition. In the 0 and 2 h groups the HG containing developing serum was removed after 3 h of pre-complement incubation, complement added and the HG returned to the trays for a further hour. The 3 h group received developing serum and complement together.

† MIG (homophile RF) plaques were of poor quality and the count was lower than usual. This may have been the result of the unconventional method of developing serum 'addition'.

IgM plaques. Not illustrated in Fig. 2 are several titrations all of which show that a developing serum optimum concentration for a particular anti-μ serum, is identical for reverse (total IgM), heterophile RF (BIG; Rat IG) and homophile RF (MIG) plaques.

Dose response

Figures 3 and 4 show, respectively, that heterophile and homophile RF 'responses' to LPS injection are dose-dependent. The maximum is at about 25–50% of the lethal dose in CBA mice. Figure 4 also shows that a homophile RF response expressed as a fraction of the total IgM forms a plateau over a dose range from 0·03–30 μg: below 0·03 μg there is a significant drop in this fraction.

Time response

LPS activates a wide range of IgM, and to a more limited degree IgG, responses in CBA mice. Some examples are given in Figs 5 and 6 where it can be seen that the peak response is reached on days 3 or 4.

Target antigen

It has been shown that in most mice there is a high level of IgM-PFC synthesizing antibody against cryptic antigens common to most mammalian erythrocytes. These antigens can be revealed by treatment of syn- and heterogeneic RBC with proteolytic enzymes such as bromelain (Steele & Cunningham, 1978). Since heterophile-RF plaques might be artefacts caused by an analogous revelation of cryptic antigens during the chromic-chloride coupling procedure, an assessment of the similarities of SRBC, BIG-coated SRBC (RF) and bromelain-treated SRBC (BrS) was made. Figure 6, which illustrates one result of this investigation, shows that BrS behave more like SRBC than RF, in that rabbit anti-μ 'developing' serum develops the RF plaques but inhibits both the SRBC and the BrS PFC. Furthermore the peak RF response is at least a day later than the anti-BrS response. This difference between RF(BIG) and BrS responses is further underlined in the experiments summarized in Table 3, where it can be seen that rabbit anti-(mouse)-μ inhibits BrS and potentiates RF, whereas free BIG (160 μg/ml)

Table 2. Rabbit antibody against either chain of the IgM molecule can act as a developer of RF-plaques

Serum*	PFC/10^6 spleen cells (±S)§ SRBC	BIG
Nil (control)	94 (18)	995 (679)
NRS†	61 (30)	785 (485)
Rabbit anti-(mouse)-μ	32 (18)	2987 (754)
Rabbit anti-(mouse)-μ through immunoadsorbent‡ column	N/D	823 (433)
Rabbit anti-(mouse)-κ	N/D	1714 (499)
Rabbit anti-(mouse)-λ_1	N/D	706 (396)

* Rabbit antisera specific for α-, γ_{2a}-, γ_3-, δ- and J-chains failed to develop any homo- or heterophile RF plaques. However, anti-γ_1 developed some anti-BIG plaques, which reach a peak 5 days after LPS and which are not inhibited by goat anti-(mouse)-μ, but no 'homophile RF' plaques were detected after anti-γ_1, development. An apparently specific anti-γ_{2b} serum had a slight developing effect on heterophile RF (IgM anti-BIG) plaques.

† Normal rabbit serum at the same dilution as the anti-μ serum.

‡ Rabbit anti-(mouse)-μ serum passed through an immunoadsorbent column of Sepharose 4B- 6 carbon spacer - TEPC 183 myeloma protein, prepared according to the maufacturer's instructions (Pharmacia).

§ Pool of five spleens from CBA males 4 days after 10 μg LPS injected i.p. Four estimations per point.

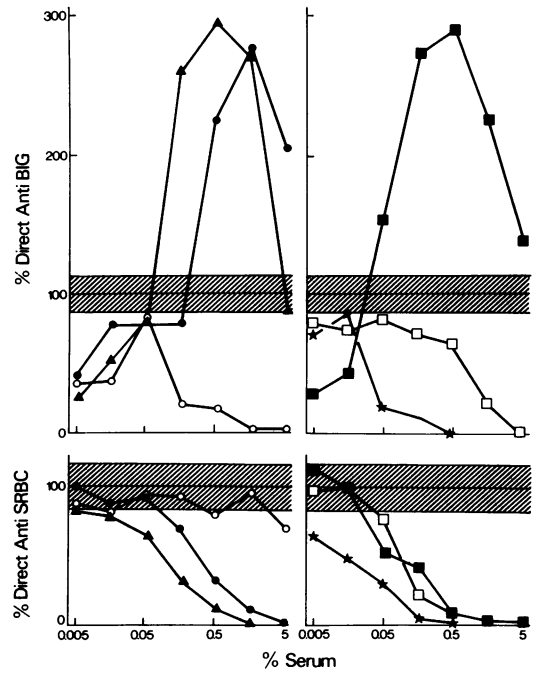

Figure 2. Titration of anti-(mouse)-μ sera for developing and/or inhibiting various sorts of IgM plaques. The upper pair of graphs are of heterophile RF (BIG) plaques and the lower pair are of plaques against uncoated SRBC. The hatched areas represent the mean and standard deviation of four estimates of plaque numbers on slides without any developing serum. In the left half of the figure, ● and ▲ are two rabbit antisera (RP-7 and RMEP-90 respectively) and ○ is a normal rabbit serum (NRS-1). In the right half ■ is a guinea-pig IgG2 preparation and □ is an IgG1 preparation from the same antiserum. After fractionation, these fractionated immunoglobulin preparations were adjusted to a volume equivalent to that of the original serum. ★ is an IgG preparation from a hyperimmune goat antiserum. Each point represents the arithmetic mean derived from a pool of five CBA spleens 4 days after LPS injection.

added to the plaquing medium strongly inhibits RF but has no effect on BrS plaques.

Both sheep and horse RBC can be successfully used as the target vehicle for coating with BIG or MIG for the detection of heterophile (Table 4) or homophile RF plaques. It therefore seems unlikely that the detection of RF plaques is an artefact of chromic-chloride treatment of sheep RBC.

Several commercially available ethanol fractionated (Fr.II) mammalian IgG preparations have been successfully coated onto SRBC by chromic chloride for use as target cells for the detection of RF plaques. These results are summarized briefly in Table 5. In addition it had been found that with most sheep the RBC should be used for coating between 10 and 30 days after bleeding. All sheep used in these experiments had been selected as possessing RBC which elicited high primary responses *in vitro* in Mishell-Dutton (1967) cultures.

Histology

Slides for histological examinations were fixed in 2·5% gluteraldehyde made up in phosphate-buffered saline (PBS) pH 7·2 for 10 min before being washed and dried. After staining with haematoxalin and eosin or methyl green pyronin, plaques were examined microscopically to determine the type of nucleated cell at their centre. Since reverse and MIG-RF PFC comprise such a relatively high proportion of spleen cells (up to 10% of cells of lymphocyte size after LPS), plaque assays carried out using spleen cells at high dilution (1 spleen in 10 litres) result in well over 95% of all plaques containing a single nucleated cell. The majority of these nucleated cells are small or medium lymphocytes

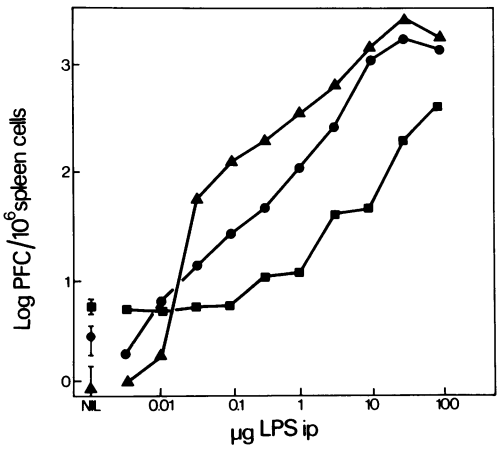

Figure 3. Dose-response curves for heterophile RF and direct anti-SRBC plaques 4 days after LPS injection in CBA males. Each point is based on an enumeration of PFC in a pool of four spleens and are therefore effectively arithmetic means. The nil controls, where the four spleens were assayed individually were an exception: the symbol and bars represent an arithmetic mean and one standard error. ▲, Indirect μ anti-BIG; ●, direct anti-BIG; ■, direct anti-SRBC.

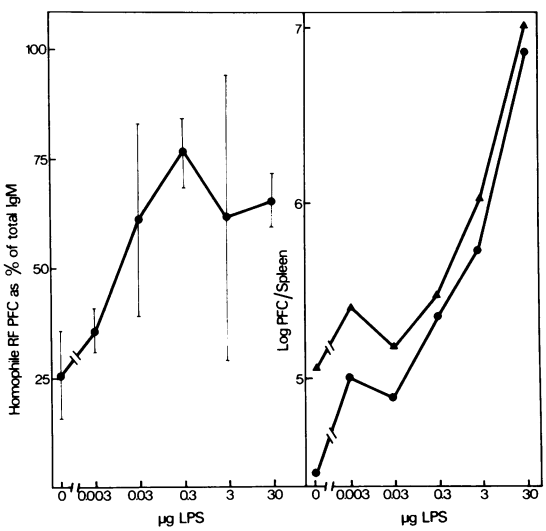

Figure 4. Dose-response curves for homophile RF (MIG) (●) and reverse (total IgM) (▲) plaques are in the right half of the figure. On the left is a representation of the homophile RF as a fraction of total IgM response: arithmetic means and one standard error are represented. The points on the right hand side of the figure are arithmetic means of individual estimates of four mice 4 days after the injection of the indicated doses of LPS, none of the error bars were larger than twice the size of the symbols and have therefore been omitted.

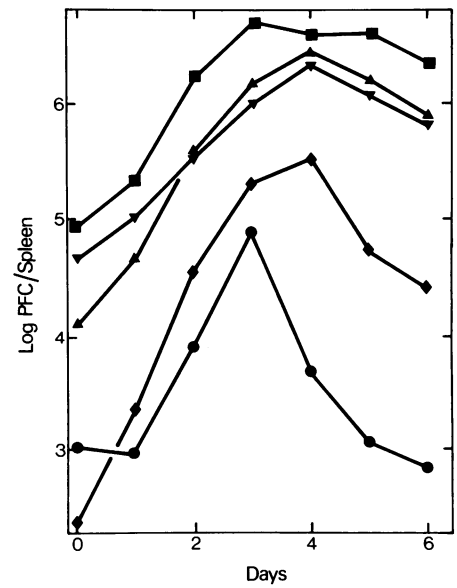

Figure 5. A time–response curve after the injection of 20 μ LPS i.p. into CBA mice. Each point is derived from a pool o four spleens. In MIG (Hi) the MIG was coupled to SRBC i the standard cold chromic chloride technique using a protei concentration of 1 mg/ml for the stock solution: MIG(Lo was at 0·5 mg/ml. Although many of the data in this figur were included in an earlier preliminary publication (Dresse 1978b) they are repeated here to facilitate comparisons. Tha RF and total IgM plaque responses peak 4 days after th injection of LPS has been observed in many subsequen experiments. ●, SRBC direct; ▲, BIG; ▼ MIG (Hi); ▲, MI (Lo); ■, total IgM.

with only a few (< 5%) which could be large lympho cytes or monocytes.

DISCUSSION

The pattern of appearance and disappearance of cell secreting RF in mice after the injection of LPS, i similar to a conventional IgM PFC response after th injection of antigens such as SRBC or DNP-Ficoll. I addition to being time-dependent, responsiveness i also dose-dependent. A difference between the 'speci fic' response to antigen and the 'non-specific' respons to LPS is that with the former up to 2% (10% with anti TNP responses) of all IgM-secreting cells may be pro ducing specific antibody at the peak of the response whereas in the non-specific response, homophile RF producing cells may exceed 75% of the total number o

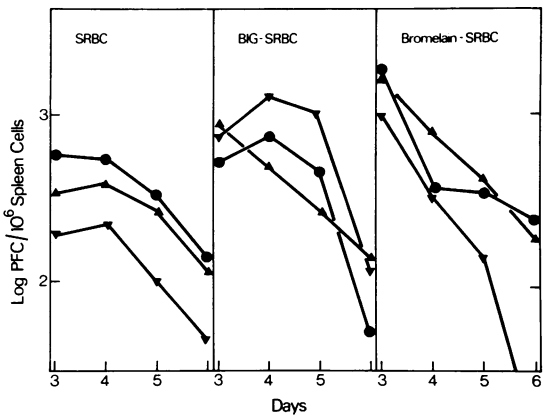

Figure 6. The direct IgM plaques against SRBC and bromelain-treated SRBC behave differently to heterophile RF (BIG) plaques. Unlike the other two where inhibition is observed, heterophile RF plaques are developed by a rabbit anti-(mouse)-μ serum. A further difference lies in the timing of the heterophile RF (peaks day 4) and the bromelain-SRBC (BrS) response which reaches a peak on day 3 after the injection of 20 μg LPS. The peaking of the BrS responses on day 3 was confirmed in two subsequent experiments. Developing serum: ●, direct (nil); ▲, NRS; ▼, R anti-μ.

IgM-secreting cells. In our experience so far, all heterophile RF responses have consisted of a lower fraction of total IgM than homophile RF responses, although, interestingly, heterophile RF measured using rat-IG coated target cells gave higher values with CBA mouse spleen cells than any other xenogenic IG.

Homophile (MIG) RF and reverse (total IgM) plaques have in common a dependence on development by an anti-IgM serum. In this respect mouse RF PFC differ from published accounts of human RF PFC (Vaughan, Chihara, Moore, Robbins, Tanimoto. Johnson & McMillan, 1976; Taylor-Upsahl, Arbrahamsen & Natvig, 1977), where IgM RF PFC appear

Table 3. Confirmation that heterophile RF (BIG) PFC and plaques formed against bromelain-treated SRBC are different.

Development with anti-μ	Free BIG	PFC/10^6 spleen cells					
		Day 3			Day 4		
		Target cells			Target cells		
		SRBC	RF	BrS	SRBC	RF	BrS
No	No	91	424	306	66	573	94
No	Yes	53	126	278	46	8	68
Yes	No	31	1076	37	34	1927	16
Yes	Yes	41	43	24	26	0	22

Assay of pool of five CBA spleens 3/4 days after the i.v. injection of 25 μg LPS. Free BIG added to gel on slide at a concentration of 160 μg/ml. RF and BrS (bromelain-treated SRBC) PFC counts corrected for PFC against untreated SRBC in equivalent conditions.

Table 4. BIG can be coated onto either sheep or horse RBC to form target cells for the detection of RF-plaques

Activator	BIG coated target RBC	Inhibitor (IgG)	PFC/spleen‡ μg inhibitor/ml	
			NIL	150
BAP*	Sheep	Bovine	24,075	3,150
	Horse	Bovine	83,700	2,025
LPS†	Sheep	Bovine	17,175 (206,000)	0
		Human	15,975	18,300
	Horse	Bovine	66,600 (264,000)	4,425
		Human	77,550	109,650

* 100 μg alum precipitated BIG plus 2 × 10^9 *B. pertussis* i.p.

† 50 μg LPS i.p., all assays 3 days after injection except for results in parenthesis which are after 4 days.

‡ Pooled spleen cells from five CBA mice.

Table 5. Different specificities of heterophile RF target cells

IgG	Target antigen (on SRBC) Source	Fraction†	PFC/spleen*
Bovine	Armour	Fr II+DE	205,000
	Serum	DE	<1,000
Canine	Pentex	Fr II	13,200
Rat	Pentex	Fr II	52,500

* Pool of four spleens from 8 month old CBA males injected 4 days previously with 20 μg LPS i.p.

† Cohn ethanol fraction II (Fr II) and ion exchange chromatography on DEAE cellulose (DE). Except for rat IgG used at 0·5 mg/ml all preparations were at a concentration of 2 mg/ml for use in the chromic chloride coupling procedure. Human IgG (Fr II) coated SRBC tended to lyse spontaneously on the addition of guinea pig complement. Bovine $F(ab')_2$ (from Fr II BIG) coated SRBC were negative. Sheep IgG (Fr II) and mouse IgG (Fr II; MIG) from C_{57}, CBA and F_1 hybrid of these strains have been used successfully on several occasions. Additional mild acid treatment of MIG results in an improvement of the quality of homophile RF plaques.

as direct plaques, The heterophile RF (BIG) plaques which are described in this paper are partially direct since they become visible to some degree after the addition of guinea-pig complement alone. Addition of an anti-μ developing serum at the start of incubation, results in a three- to five-fold increase in countable heterophile RF plaques and also in a marked improvement in the quality of the plaques themselves.

Rabbit anti-(mouse)-μ serum acts as a developer for RF and reverse (IgM) plaques but inhibits what are normally regarded as direct plaques. Analogous goat antisera inhibit all types of IgM plaques. It is assumed that the bulk of rabbit IgG is of an isotype equivalent to IgG2 in other species (Rodkey & Freeman, 1969) and that most IgG in hyperimmune goat antiserum is of the IgG1 subclass. Rabbit IgG (IgG2) can fix guinea-pig complement (C) when complexed with an antigen at a sufficiently high concentration to give a high incidence of pairs of contiguous IgG molecules (Humphrey & Dourmashkin, 1969), whereas IgG1 antibody molecules possibly lacking a C binding site are less capable of initiating C fixation. It is possible that there may be sub-class differences in the initiation of C action in the classical and alternative pathways (Spiegelberg, 1974).

This explanation of the difference between rabbit and goat anti-μ sera, was tested using a guinea-pig antiserum, where the IgG1 and IgG2 subclasses are readily separable by ion-exchange chromatography. It was possible to demonstrate that IgG1 anti-(mouse)-μ inhibits (or totally fails to develop) both RF, reverse IgM and direct IgM plaques, whereas the IgG2 fraction developed RF and reverse IgM plaques. Like the rabbit anti-μ serum, the guinea-pig IgG2 anti-μ inhibited direct plaques against SRBC and TNP. The simplest explanation of the phenomenon, which suggests that there are two sub-classes of IgM, one which fixes C and the other which does not (Plotz *et al.*, 1968), may provide a partial explanation. The numerology of direct IgM PFC against antigens such as SRBC and TNP, taken together with indirect (μ-developed) reverse IgM PFC, makes it clear that this cannot be the only mechanism responsible for the observed phenomenon.

An alternative explanation is that a degree of cross-linking is necessary to raise the avidity of a RF/IgG complex above an operational threshold below which C fixation and consequent target cell lysis is not possible. However, IgG1 (anti-μ) antibody molecules lack their own C binding sites and while cross-linking the RF/IgM molecules, sterically block the complement binding sites in the IgM. In contrast, IgG2 anti-μ antibody compensates for the sterically blocked C binding sites of the RF molecules through possession of their own sites. This may be similar to the experiment of Henney & Ishizaka (1968) who demonstrated that a complex of rabbit IgG (anti-chicken IgG) with chicken IgG fixed C whereas the reciprocal complex (with rabbit IgG as the antigen) failed to do so. Presumably the chicken IgG (antibody) which does not have a binding site for guinea-pig complement, sterically blocked the site on the rabbit IgG molecule.

The high proportion of homophile RF-producing cells is of interest from several points of view. RF may be related to pathological conditions such as rheumatoid arthritis or it may play a normal physiological role in controlling immune responsiveness perhaps in a manner analogous to antibody feedback (Uhr & Möller, 1968; Henry & Jerne, 1968; Rowley, Fitch, Stuart, Köhler & Cosenza, 1973), or the currently popular version of 'feedback' which involves idiotypic specificities as key links in a network of feedback loops (Jerne, 1974). A teleological interpretation of the theory of evolution encourages a search for the advantages of (or reasons for) universally high rheumatoid factor responsiveness. In this context high levels of autoreactivity (Steele & Cunningham, 1978; Dresser, 1978b) may be of some relevance to concepts of the generation of B-cell diversity, perhaps in a way analogous to the role of the MHC in the generation of T-cell diversity, postulated by Jerne (1971). A more mundane reason may be that RF acts as an amplification step for complement fixation to IgG/antigen complexes which in themselves lack complement binding sites (IgG1). Although we conceived this mechanism in terms of the classical pathway (Dresser & Popham, 1976), it has been pointed out to us by several colleagues that the alternative pathway (Götze & Müller-Eberhard, 1976) cannot be excluded from this hypothesis.

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