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IgM rheumatoid factor as a source of non-specificity in murine anti-allotype sera

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Summary. Anti-allotype sera have shown a breakdown of the expected specificity especially when used for the development of haemolysis of fragile target erythrocytes. IgM anti-IgG rheumatoid factor has been shown to be the source of non-specificity. Removal or destruction of IgM in an anti-allotype serum restores the expected specificity.

INTRODUCTION

For practical immunological purposes an Ig-allotype is a serologically detectable polymorphism of immunoglobulin molecules (Herzenberg, McDevitt & Herzenberg, 1968). Consequent of current dogma it is often assumed that reciprocally raised anti-allotype sera will be completely specific when used to distinguish between the auto-allele and its homologue. For example the antibody of an anti-*b* serum, raised in CBA ($Ig^{a/a}$) mice immunized with C57 ($Ig^{b/b}$) IgG, should bind to C57 IgG but not bind at all to CBA IgG molecules. In so far as this remains true, allotypic markers can be used to trace the cellular origin of Ig products. It was therefore somewhat disconcerting to encounter an apparent breakdown in specificity of reciprocally raised anti-allotype sera (Dresser, Keeler & Phillips, 1976).

Precipitin assays and the development of indirect plaques in haemolytic plaque assays employing unaltered sheep red blood cells (SRBC) as the target antigen, confirmed the expected specificity of anti-allotype sera. When, however, plaque assays were carried out using haptenized-SRBC or the IEF (isoelectric focusing)-overlay method (Phillips & Dresser, 1973a, b) was used with either unaltered or haptenized-SRBC, it soon became clear that there was a considerable degree of non-specific development of haemolysis: it is thought that increased fragility of the target cells in these situations contributes to the increased sensitivity to non-specific development. In investigating the phenomenon we have identified the source of non-specificity as an IgM molecule which binds IgG especially IgG antibodies bound to antigen: this IgM molecule has features in common with an M rheumatoid factor. The expected and desired specificity of anti-allotype sera can be restored by procedures which destroy (controlled reduction and alkylation) or remove (gel filtration; ion exchange chromatography) IgM from the anti-serum.

MATERIALS AND METHODS

Sheep red blood cells (SRBC) which were obtained either from Burroughs Wellcome Ltd, or from our own sheep (S-23), were stored in Alsever's solution and prior to use were washed three times in Hanks solution.

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Bordetella pertussis (B.p.) was obtained from Mrs J. Dolby (Lister Institute, Elstree, Herts, WD6 3AX). Other reagents were DEAE–cellulose (DE52; Whatman) and Ultragel ACA22 (LKB).

At the time when the bulk of the experiments described here were in progress, some mice at the NIMR were bred in specific pathogen free (SPF) conditions and some in conventional conditions: where relevant, the origin of the mice is indicated in the results section. Mice of strains CBA/Ca-NIMR (Syn. CBA/H) ($Ig^{a/a}$) and C57Bl/6-NIMR ($Ig^{b/b}$) were used in these experiments together with an interim twelfth backcross line of CBA (congenic $Ig^{b/b}$) (CBA b12) bred in conventional conditions: this line has now been discarded and replaced by a CBA b20 line which is bred in SPF conditions.

Sera

Anti-allotype sera were raised by injecting, *Bordetella pertussis* organisms (B.p.) complexed with specific antibody of one strain, intraperitoneally (i.p.) into the other strain (Dresser & Wortis, 1967). For example a CBA anti-B.p. serum is incubated for 30 min or so with B.p. at room temperature, the B.p. is then washed two or three times by centrifugation and resuspension of the bacteria in fresh medium. The B.p.–antibody complex is now injected i.p. into C57Bl mice at fortnightly intervals on four to eight occasions. The C57Bl mice are then test bled and their sera run against CBA serum in a double diffusion in agar (Ouchterlony) plate. Positive C57 animals are caged together and bled to form a pool of anti-*a* serum (e.g. A-44). An anti-*b* serum (e.g. A-43) is prepared in a reciprocal manner. In addition to allotype specific antibodies these sera contain immunoglobulin which have arisen as a consequence of the repeated injection of B.p.

Individual serum samples from mice immunized with SRBC were stored at $-20°$. Necessary details of how these sera were raised are given in the results section where appropriate.

Target erythrocytes

The haemolytic (Jerne) plaque assay on slides and the IEF overlay assay were carried out by procedures which have been described in detail (Phillips & Dresser, 1973a, b; Dresser 1978a). For both these assays washed SRBC are used in the usual manner. Standard plaquing methods were used in preliminary experiments (Table 1) where IgG anti-NIP responses were measured (Dresser 1978a).

The coating of SRBC with allotype specific Ig was carried out by incubating washed erythrocytes with anti-SRBC sera from CBA or C57 mice and subsequently washing these cells to remove un- and lightly bound antibodies (see Table 3).

Fractionation or treatment of anti-allotype sera

Gel filtration of sera absorbed with SRBC was on a 900×25 mm column of either Biogel 1.5A (Calbiochem) or ACA22 (LKB) in a physiologically isomolar phosphate buffered saline (pH 7·8) containing 2 mM EDTA (SSE: Dr Sayaka Utsumi, personal communication). Fractions were concentrated to a volume equivalent to that of the original serum, using an Amicon diaflo and then by pressure dialysis against physiological saline. In some experiments the fractions were reabsorbed with SRBC.

DEAE chromatography was carried out using Whatman DE52 in a starting buffer of 0·01M Tris/HCl pH 8·1. Globulins were first prepared by precipitation from whole serum in half-saturated ammonium sulphate at 4°, followed by equilibration against the starting buffer. Essentially all the globulin fraction binds to the DE52 in this buffer: two elutions were made by stepping up the molarity of the elution buffer through the addition of NaCl to 0·02 M and then 0·06 M respectively. Fractions were absorbed, concentrated and dialysed as above. Whole serum was reduced and alkylated by making it to 0·01 M dithiothreitol (Calbiochem) and 0·1 M Tris/HCl pH 8·6. After half an hour at 22° free -SH were alkylated by the addition of an appropriate amount of iodoacetamide in 0·1 M Tris/HCl at pH 7·6.

Iso-electric focusing overlay technique

In our experiments this assay was usually carried out by first focusing antibodies specific for SRBC and then visualizing these molecules by overlaying the acrylamide gel containing the focused antibody with 3 ml of an agarose gel of Eagle's MEM containing a mixture target erythrocytes (250 μl of 15% SRBC) and anti-immunoglobulin developing serum, followed 2 h later by the addition to the surface of the gel of 10% fresh guinea-pig serum diluted in Hanks's solution, as a source of complement. This results after incubation at 37°, in visible and discrete zones of lysis in the layer of erythrocytes. An anti-immunoglobulin developing serum is needed since in the conditions of these experiments IgM does not focus and the trace amounts of direct haemolysis which are observed are restricted to very high titre IgG (γ_{2a}) antibody. The developing serum which was used in these experiments was speci-

fic for IgG (γ_1 and γ_{2a}) and was used at an optimum concentration derived empirically by titration in a plaque development system (Dresser 1978a).

RESULTS

Although in the past anti-allotype sera have been used to develop indirect (SRBC) plaques (Taylor, Wortis & Dresser 1966; Wortis, Dresser & Anderson, 1969), a failure in the expected specificity was not observed. More recently, however, when SRBC coated with NIP-(4-hydroxy-5-iodo-3-nitrophenacetyl-) were used as target erythrocytes a breakdown of anti-allotype specificity was observed. An example of this is given in Table 1, where it can be seen that not only does the 'wrong' anti-allotype serum develop indirect plaques but serum from mice immunized with B.p. alone also has a developing effect. It seems likely that the non-specificity phenomenon only becomes apparent when the target erythrocytes are unusually fragile or the epitope/antibody concentration very much increased, since SRBC uncoated or coated with DNP-, did not show any non-specificity of development of haemolytic plaques.

At about the same time that we started to use anti-allotype sera to develop IEF-overlay plates (Phillips & Dresser, 1973a, b), we were disconcerted to discover that in many circumstances the 'wrong' anti-allotype serum developed indirect spectrotypes. For example, when CBA anti-SRBC IgG antibodies were focused in replicate plates and individual plates were overlaid with target cells mixed with various anti-allotypes and control sera, we found that CBA anti *b* serum ('wrong') was just as effective as C57 anti *a* ('correct' or expected) at developing bands of lysis (spectrotypes). Figure 1 is an illustration of such an experiment, where in addition to 'right' and 'wrong' anti-allotype sera, we have used anti-B.p. and normal mouse sera together with a class specific rabbit anti-(mouse)-IgG.γ_1 serum. Figures 1 and 2 illustrate results obtained with sera from lethally irradiated CBA mice ($Ig^{a/a}$) adoptively immunized with immune spleen cells from CBAb12 ($Ig^{b/b}$) mice. Similar results are obtained with sera from actively immunized C57Bl/6 ($Ig^{b/b}$) mice (Fig. 3). It will be noted in Figs 1,

Table 1. A breakdown of anti-allotype specificity in the development of indirect anti-NIP haemolytic plaques

Origin of primed and boosted spleen cells (Ig phenotype of mice)	Corrected PFC/10^6 spleen cells*				
	Direct	Developed by anti-allotype serum		Developed by anti-*B. pertussis* serum	
		IgG (γ2a)		b	a
	IgM	*b* anti-*a*†	*a* anti-*b*‡	Anti-B.p.§	Anti-B.p.¶
CBA (*a*)	59	32	104	116	66
CBAb12 (*b*)	95	124	351	265	129

* IgM (direct) have PFC against uncoated target cells subtracted and the developed (IgG2a) have the value of the direct assay subtracted. Arithmetic means of four animals per group.

† A-26: C57Bl/6 (b/b) anti-*a* (together with anti-*B. pertussis*).

‡ A-40: CBA (a/a) anti-*b* (also with anti-*B. pertussis* activity).

§ A control anti-*B pertussis* serum raised in C57Bl/6 mice.

¶ Similar to § but raised in CBA mice.

Target cells were coated with NIP-using a Fab fragment of a rabbit anti-SRBC IgG ($NIP_{5\cdot8}$ Rab. Fab.). Similar results, but with lower absolute values were obtained when NIP-whole chicken gamma globulin (from antiserum against SRBC) was used to haptenize the target cells. The mice were primed with 100 μg alum precipitated $NIP_{3\cdot8}$ bovine IgG plus 2×10^9 *B. pertussis* organisms injected i.p. and the boost (5 weeks after priming; 8 days before assay) was 40 μg of soluble $NIP_{3\cdot8}$ bovine IgG.

Parallel experiments using SRBC or DNP- failed to reveal significant non-specific development of indirect plaques.

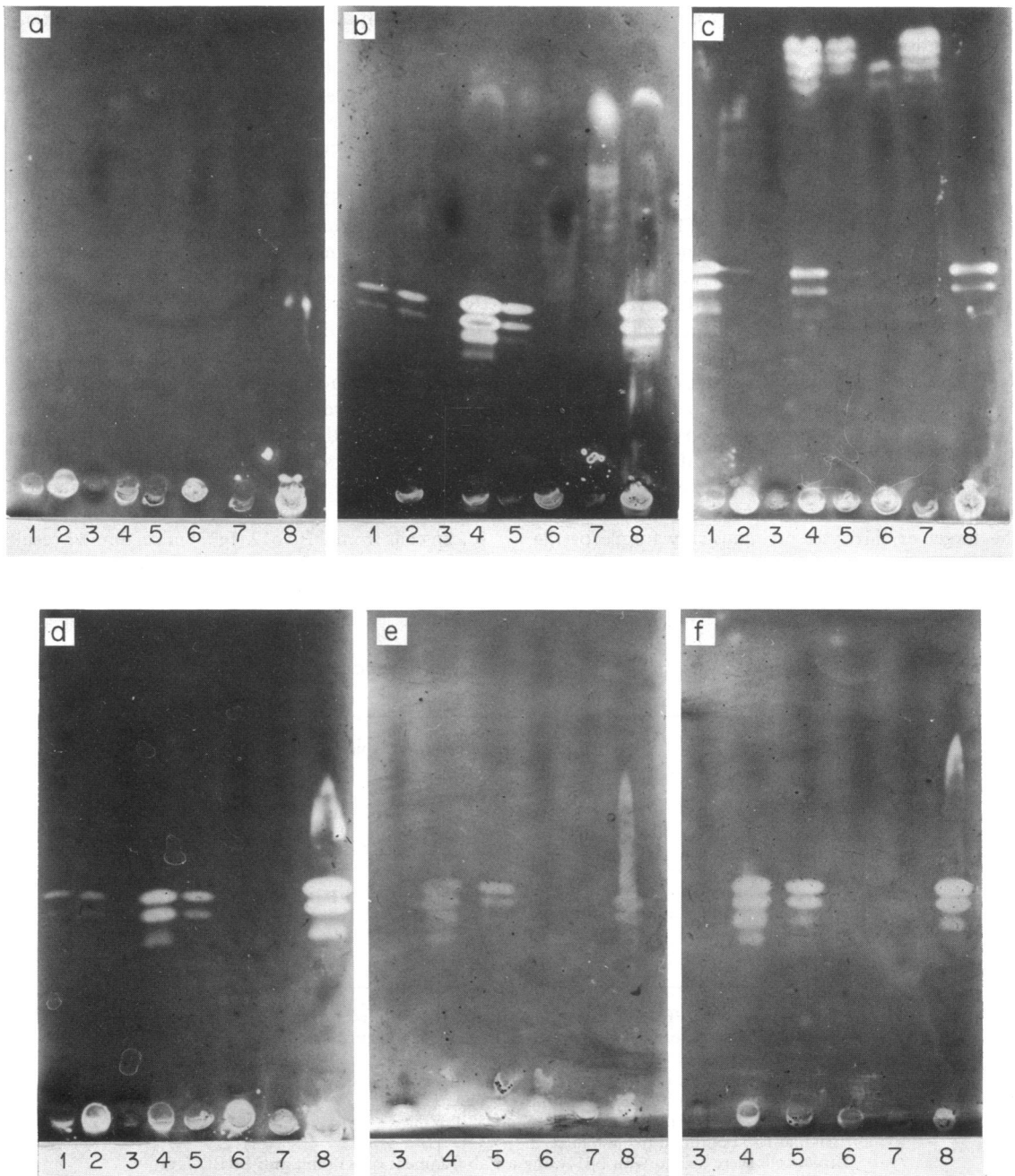

Figure 1. Photographs of IEF-overlay plates on which sera from irradiated recipient (*a* allotype) mice adoptively immunized with spleen cells from CBA b12 (*b* allotype) mice which had previously been actively immunized against SRBC: individual sera are numbered from 1 to 8. (a) is a direct plate with no developing serum; (b) had 15 μl of CBA anti-*b* serum added to the 3 ml of overlay medium; (c) had a specific rabbit-anti-(mouse)IgG(γl) serum added; (d) received C57 anti-*a*; (e) C57 anti-B.p. and (f) CBA anti-B.p. At present we have no explanation for the relative difference in intensity of development of spectrotypes in certain individual anti-SRBC-sera (1 and 4) by rabbit developing serum (c) or by mouse sera (b and d).

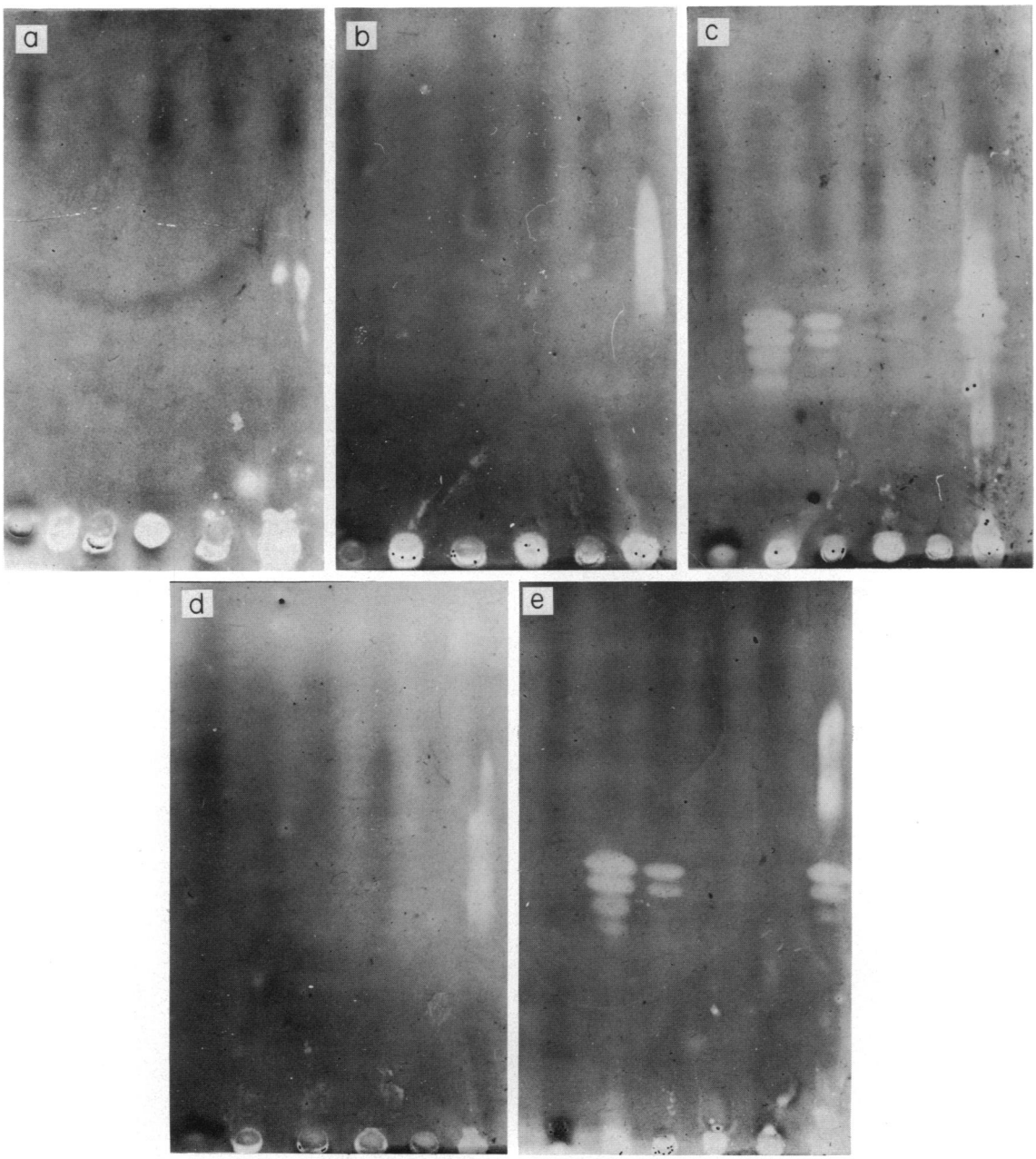

Figure 2. Similar to Fig. 1 using samples labelled 3–8 (1 to r): (a) direct with no developing serum; (b) 20 μl serum from non-immune SPF CBA mice added to the 3 ml of overlay medium; (c) serum from non-immune conventional CBA mice; (d) with serum from non-immune SPF C57Bl 6 mice; (e) with serum from non-immune conventional C57Bl 6 mice.

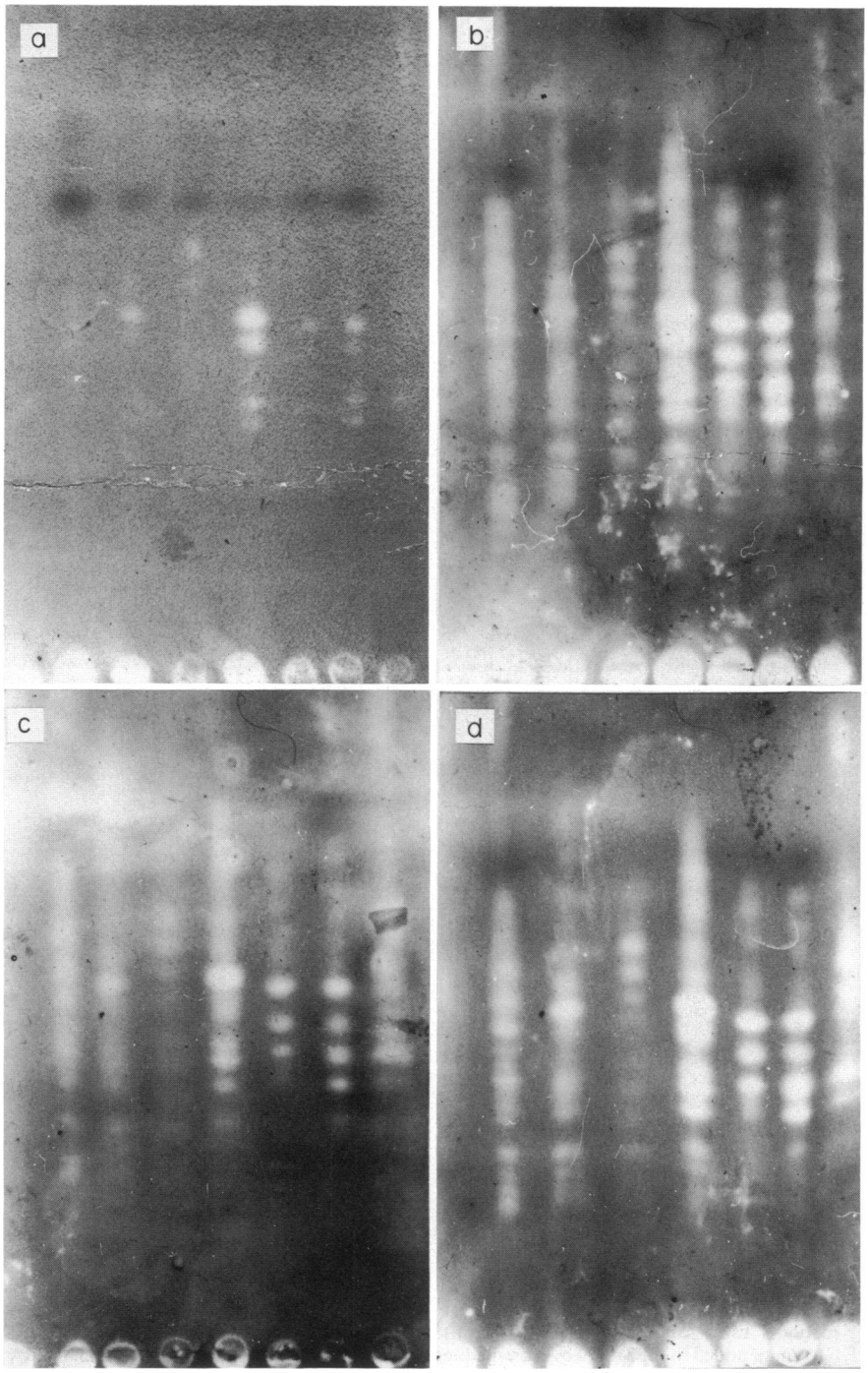

Figure 3. Samples from C57Bl 6 mice immunised with 4×10^7 SRBC i.p., bled on day 10: (a) direct plate with no developing serum added; (b) serum from non-immune conventional CBA mice; (c) serum from SPF CBA mice injected with 10 μg pneumococcal polysaccharide (S3) 5 days before bleeding; (d) SPF CBA mice injected with 50 μg LPS 5 days earlier.

Table 2. Fractionation of anti-allotype developing sera to identify and remove the source of non specificity: analysis by IEF-overlay using SRBC target cells

Developing serum: fraction or treatment	Spectrotype development*	
	(*a*)†	(*b*)†
Direct, no serum added	−	±
A44 (*b* anti-*a*), whole serum	++++	++++
Gel filtration 19S	++	+++
7S	++++	±
DE 52 0·02 M	++++	±
0·06 M	++++	++++
0·01 M DTT	++++	−
A43 (*a* anti-*b*), whole serum	+++	+++
Gel filtration 19S	‡	‡
7S	±	+++

* Subjective assessment of the development of spectrotypes made from photographs similar to those illustrated in Figs 1–3.
† Serum samples (2–3 μl) from four *a* allotype and four *b* allotype mice immunized with SRBC 10 days previously, were focused on each plate.
‡ The 19S fraction precipitated spontaneously.

2 and 3 that in addition to development by 'wrong' anti-allotype sera, both *a* and *b* anti-B.p. sera will develop indirect spectrotypes. In contrast, sera from mice maintained in specific pathogen free (SPF) conditions, failed to develop any spectrotypes, although sera from mice bred in SPF conditions but subsequently kept for 3 or 4 weeks in conventional conditions will develop indirect spectrotypes.

We tested our initial hypothesis that non-specificity was the consequence of antibody to B.p. by attempting to absorb out anti-B.p. activity in anti-allotype sera. Addition of B.p. to anti-allotype sera followed by its removal by centrifugation failed to remove any of the non-specific developing activity. Similarly the use of columns of B.p. coupled to Sepharose 4-B also failed to remove it. Consequently, we modified our hypothesis to propose that non-specificity was due to globulins appearing in the serum as a consequence of the (random mitogenic or polyclonal) stimulation of lymphoid cells by B.p., LPS or infection by unspecified enteric bacteria in the conventional animal house, which could react with IgG antibodies complexed to antigen. This would be IgM and analogous to rheumatoid factor.

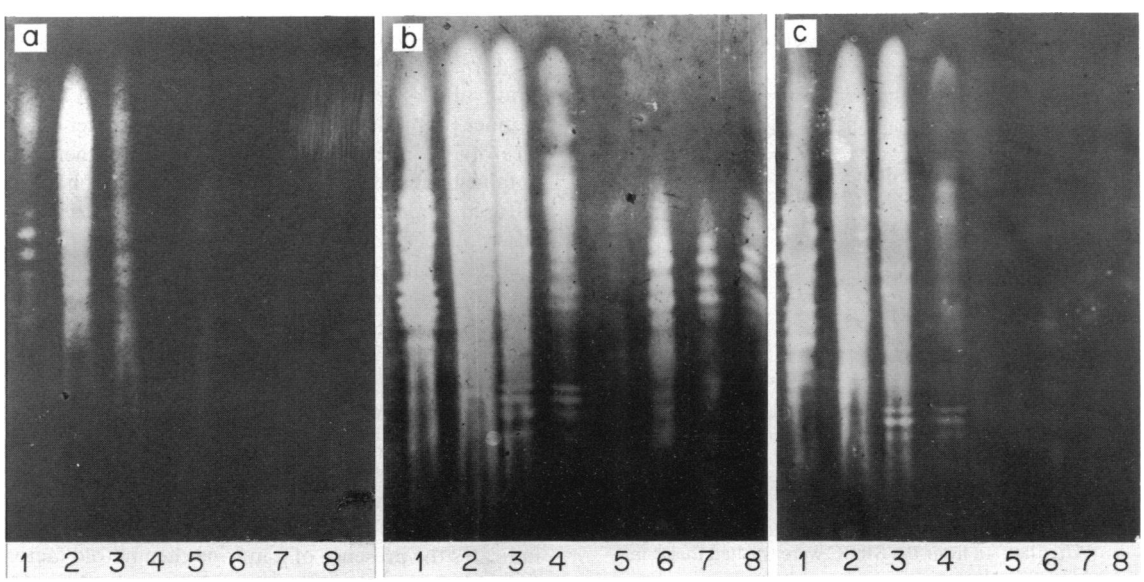

Figure 4. Samples 1–4 were from CBA (a/a) mice and samples 5–8 were from CBA b20 (b/b) mice, all immunized with 1×10^8 SRBC i.p.: (a) direct plate; (b) 15 μl of C57Bl 6 anti-*a* serum added to the overlay medium; (c) an equivalent amount of C57Bl 6 anti-*a* serum after passage down a goat anti-(mouse)-μ (IgG preparation)- arm-Sepharose 4B column, to specifically remove IgM from the anti-(*a*)-allotype serum.

The modified hypothesis was tested by analysing the non-specific developing activity of anti-allotype sera after fractionation by gel filtration, by ion exchange chromatography or by reduction and alkylation in conditions calculated to destroy IgM but leave IgG relatively unscathed. The results are subjectively summarized on a semi-quantitative basis in Table 2, from data such as that illustrated in Fig. 1–3. It can be seen in this table that specific development is by 7S; very low molarity eluate from DEAE; and DTT resistant material. In contrast, non-specificity was caused by 19S; medium to high molarity eluate from DEAE; and DTT sensitive material (IgM).

Finally, the μ-specificity of the material causing non-specific development was demonstrated by removing such activity when a crude antiallotype serum was passed down an anti-μ affinity column (IgG of a goat-anti-(mouse)-μ on Sepharose): the result of this experiment is illustrated in Fig. 4. The efficiency of this anti-μ affinity column was greatly increased by inserting a spacer molecule between the agarose matrix and the antibody molecules (Sepharose-arm-anti-μ) using the methodology of Cuatracasas (1970) and Cuatracasas & Parikh (1972).

We have observed that with only one exception in many experiments γ_{2a} is the only IgG subclass to be 'non-specifically' developed by rheumatoid factor.

Table 3. Rheumatoid factor producing cells in mice making anti-allotype antibody

Spleen cells*	Target cells†	mean PFC/spleen
Day 3	SRBC	132
	b-SRBC	6,072‡
	a-SRBC	58,344
	a-SRBC	41,712§
Day 6	SRBC	396
	b-SRBC	132
	a-SRBC	67,188
	a-SRBC	48,180§

* Time in days after a second boost of C57 mice with a complex of CBA anti-B.p. on B.p. Pools of three spleens in each group.

† Target cells prepared by adding 3 μl of CBA-anti-SRBC to each ml of 10% washed SRBC (*a*-SRBC) or 3 μl of C57 anti-SRBC (*b*-SRBC). After half an hour the SRBC were washed thrice in Hanks's solution.

‡ Non-specific plaques probably due to the presence of rheumatoid factor producing cells.

§ Late addition of anti-*b* allotype serum to test for possible development.

The exception was in the transfer experiment illustrated in Figures 1 and 2, where it can be seen that a γ_1-spectrotype is developed. This spectrotype was defined as being γ_1 by a hetero-subclass-specific (rabbit) antiserum (RP20) and its failure to be affected by (rabbit) anti-sera specific for γ_2, γ_{2b}, α and μ. The more alkaline spectrotype developed by RP20 was not developed non-specifically.

We have demonstrated large numbers of PFC, producing specific anti-allotype antibody, in the spleens of mice immunized with B.p./antibody complexes. It will be seen in Table 3 that 6 days after the last boost with immunogen, the response is almost entirely specific, whereas at 3 days post-boost there is a significant non-specific element. We believe that these PFC may be rheumatoid factor producers analogous to those demonstrated more recently (Dresser, 1978b).

DISCUSSION

Failure of reciprocally raised anti-immunoglobulin-allotype specificity in certain experiments, is clearly unsatisfactory. This is especially so if the allotype determinant is to be used as a genetic marker of the origin of a B-cell product. Having detected such non-specificity (Dresser *et al.*, 1976) we have since endeavoured to determine its cause and its cure.

Although precipitin lines can appear in Ouchterlony gel diffusion plates between certain sera from the same strain of mouse, they do so in a somewhat erratic manner (unpublished data). Some of the better examples of this phenomenon are those seen when sera obtained a few days after injection of B.p. or LPS are diffused against normal sera. A very reliable breakdown of specificity can be observed, however, when such (syngeneic) sera are tested for their ability to develop complement dependent haemolysis: this seems to be particularly true when the target erythrocytes are more fragile than usual as they are after having been haptenized with NIP- in a standard plaque assay or when used uncoated in the IEF-overlay assay.

The results show that the source of non-specificity is an IgM molecule which has binding affinity for IgG of the same and different strains. Such M- anti-G activity indicates the presence of a murine rheumatoid factor. In Table 3 it can be seen that at 3 days after boosting with B.p./anti-B.p. complex, C57 spleens contain cells which secrete rheumatoid factor. Recently very large numbers of murine rheumatoid factor producing cells

have been demonstrated (Dresser & Popham, 1976; Dresser, 1978b).

Bosma & Bosma (1974) demonstrated the presence of *a* allotype in CB17 mice, which were thought to be homozygous for *b*. This strain was the result of an intercross of the 17th backcross of *b* allotype onto a BALB/c background. They interpreted their results as support for selection of a control gene and not the basic structural gene during backcrossing. The results described in this paper do not in any way invalidate their conclusion, although they do offer an alternative explanation for their observed phenomenon.

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