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Continuous production of anti-host IgG antibodies contained in circulating IgG-anti-IgG complexes

S. A. GRACE* & C. J. ELSON *Immunobiology Group, Department of Pathology, University of Bristol Medical School, Bristol*

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Summary. Weekly injection with antibody-coated pertussis led to the chronic production of serum complexes in mice. Mice bearing one allotype, Ig^a, received spleen cells from a congenic strain bearing another allotype, Ig^b, and weekly injections with Ig^a-coated pertussis. The serum complexes from these mice and from those receiving challenges alone were separated by ultracentrifugation at neutral and acid pH on sucrose density gradients and their fractions tested for their anti-Ig^a and Ig^a content. This revealed the presence of anti-host (Ig^a) allotype antibodies in fractions from mice that had received Ig^b cells but not from mice given Ig^a-coated pertussis alone. Ig^a allotype was detected in fractions from both groups. It is considered that anti-host allotype antibodies are continuously produced but that they cannot be detected in unfractionated serum because the antibody forms complexes with Ig^a. These findings are discussed in relation to rheumatoid disease in humans.

INTRODUCTION

In recent years there has been much interest in the observation that raised levels of IgG rheumatoid factor (RF) occur in the serum and synovial fluid of patients suffering from rheumatoid arthritis (Torrigiani & Roitt, 1967; Ilter & Turner, 1973; Hay, Nineham & Roitt, 1975). This has stimulated several investigations into the role of IgG RF in the pathogenesis of rheumatoid synovitis and vasculitis. For example, IgG-anti-IgG complexes associated with decreased complement levels have been demonstrated in the synovial fluid of patients with synovitis (Winchester, Agnello & Kunkel, 1970; Hedberg, Lundh & Laurell, 1970). High serum IgG RF levels are strongly associated with the incidence of vasculitis in rheumatoid patients (Allen, Elson, Scott, Bacon & Bucknall, 1981). Moreover, in individual vasculitis patients there is a temporal relationship between serum IgG RF, anti-complementary activity, C4 levels and clinical features both in remission and relapse (Scott, Bacon, Allen, Elson & Wallington, 1981). In these patients both IgG and IgM RFs circulate together as antigen-antibody complexes (Jones, Cowley, Allen & Elson, 1981) and have the capacity to bind C3 (Elson, Scott, Blake, Bacon & Holt, 1982). Thus, IgG-IgG or IgM-IgG complexes occur *in vivo* and may be associated with the development of synovitis or vasculitis depending on whether they are localized in the joint or in the blood vessels, respectively. However, whether these complexes are involved in the initiation of the inflammatory lesions seen in rheumatoid patients is still debated.

To answer such questions we sought to manufacture persistent IgG-anti-IgG complexes in mice. The approach was to immunize mice bearing one allotype

* Present address: Department of Physiology, Godfrey Huggins Medical School, University of Zimbabwe, Mount Pleasant, Salisbury, Zimbabwe.

Correspondence: Dr C. J. Elson, Department of Pathology, University of Bristol, The Medical School, University Walk, Bristol BS8 1TD.

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(Ig^b) against IgG from a congenic strain bearing another, Ig^a. When Ig^a-primed spleen cells were transferred to congenic recipients bearing the Ig^a allotype low levels of IgG anti-host allotype briefly appeared in the recipients' serum (Grace, Elson & Coeshott, 1980). This report describes how the production of anti-host allotype antibodies was prolonged.

MATERIALS AND METHODS

Animals, cell transfers and immunization

CBA/H/Ig^a mice and the congenic strain, CBA/H/Ig^b (Micklem, Anderson, Ure & Jones, 1976; Elson, Jablonska & Taylor, 1976) aged between 3 and 6 months were used. Spleen cells from Ig^b donors were suspended in balanced salt solution and 10^8 cells were injected intravenously into each Ig^a recipient under aseptic conditions.

Immunization against Ig^a or Ig^b allotype was achieved by intraperitoneal injection of *Bordetella pertussis* (Wellcome Research Laboratories) coated with Ig^a or Ig^b antibody to pertussis, respectively. Each inoculum contained 2×10^9 heat killed organisms. Ig^b donors of Ig^a-primed cells were given 5 weekly injections of Ig^a-coated pertussis and used 1 month after the final injection.

Serum was obtained from experimental mice and stored at $-20°$ without thawing until assayed for complement consuming activity.

Guinea-pig serum was used as the source of complement and was stored at $-80°$.

Sensitized sheep erythrocytes

Sheep erythrocytes (Tissue Culture Services, Slough, Berks.) were washed three times in complement fixation test diluent (CFD) and incubated with diluted rabbit haemolysin (1/2000) for 1 hr at room temperature. The cells received a further three washes in CFD and were stored as a 10% suspension at 4°.

Assay for immune complexes by complement consumption

This was adapted for use with mouse serum from the method of Harkiss & Brown (1979). It involves the polyethylene glycol 6000 (PEG) precipitation of complexes from sera and the assay of their ability to consume complement.

Fifty microlitres of serum, 17 μl 0·1 M EDTA in borate-buffered saline pH 8·5 (BBS) and 17 μl 12·5% PEG in BBS were mixed in LP3 tubes (Luckham Ltd, Burgess Hill, Sussex) and left at 4° for 90 min. After centrifugation the supernatants were removed, the precipitates washed in 2·5% PEG and redissolved in 200 μl CFD. To 100 μl dilutions of these solutions in CFD, 100 μl of diluted guinea-pig serum (1/80) was added. The tubes were agitated and incubated at 37° for 35 min. Sensitized erythrocytes (100 μl of 0·75%) were added and the mixture incubated at 37° for 35 min. After addition of cold CFD (200 μl per tube) the tubes were centrifuged. The extent of haemolysis was determined by measuring free haemoglobin in the supernatant by absorption at 414 nm on a Unicam SP 1800 ultraviolet spectrophotometer. The percentage complement consumption by PEG precipitates was calculated using a 0% control containing complement and sensitized erythrocytes alone. The complement consumption of serum PEG precipitates was compared with that of standard dilutions of heat aggregated (30 min at 63°) purified mouse IgG and their activity expressed as microgram equivalents of aggregated mouse IgG per millilitre of serum.

Estimation of mouse anti-Ig^a allotype antibodies

Mouse myeloma protein APC5 Fc fragment was attached to microtitre plates (Linbro, Flow Laboratories, Inc.) by adding 100 μl of a solution of 50 μg/ml APC 5 Fc fragment in phosphate-buffered saline to the wells and incubating for 2 hr at 37° and overnight at 4°. After washing with phosphate-buffered saline (PBS), any free reactive sites were blocked by a further incubation for 2 hr at 37° with diluent (1% sheep serum in phosphate-buffered saline pH 7·2). Test sera, sucrose density gradient fractions or standard antibody were diluted in the wells and incubated at 37° for 2 hr. The plates were washed with diluent, incubated overnight at room temperature with ^{125}I-labelled purified anti-Fdγ antibody, washed again and counts made of the radioactivity bound to each individual well. The log amount of anti-Ig^a allotype antibodies was proportional to the label bound and was estimated by comparison to a purified standard anti-Ig^a allotype.

Estimation of IgM anti-gamma globulin activity

This was carried out as described above except that mouse IgG was attached to the plates and goat anti-μ chain was used as the radiolabelled antibody.

Detection of Ig^a or Ig^b allotypes in sucrose density gradient fractions

Microtitre plates were coated with the proteins contained in sucrose density gradient centrifugation

fractions and free reactive sites were blocked by the method described above. The presence of Ig^aIgG or Ig^bIgG attached to the plates was identified by incubation with ^{125}I-labelled purified antibody to Ig^a IgG and Ig^bIgG, respectively.

Sucrose density gradient ultracentrifugation

This was carried out as described previously (Jablonska, 1977; Jones *et al.*, 1981) using sucrose dissolved in CFD or glycine-HCl buffer (0·2 M, pH 3·0). 0·5 samples were layered onto 8 ml gradients in $1·5 \times 6·2$ cm tubes and the meniscus between the samples and the gradients blurred to prevent instability at this boundry. After centrifugation at 95,000 *g* for 18 hr at 4°, nine 0·9 ml fractions were taken from the top of the gradients using a graduated syringe. Fractions were dialysed for 24 hr to remove sucrose before testing them for complement consuming activity and their content of Ig^a or Ig^b IgG. By this method mouse (and human/IgG) is concentrated in fractions 3 and 4 and IgM in fractions 6 and 7 (Jablonska, 1977; Jones *et al.*, 1981).

Preparation of mouse IgG

IgG in normal mouse sera or ascitic fluid from mice inoculated with APC5 (IgG2a bearing the Ig^a allotype) myeloma was precipitated with 20% sodium sulphate and washed in 18% sodium sulphate. The precipitate was redissolved in 0·01 M HCl-Tris (pH 8·5) and applied to a DEAE Sepharose column (1·6 – 30 cm) equilibrated with 0·1 M HCl-Tris (pH 8·5). Protein was eluted from the column with a gradient from 0 to 0·15 M sodium chloride in 0·1 M HCl-Tris (pH 8·5). The IgG peak was concentrated and further purified by gel chromatography on ACA 34.

Preparation of APC5 Fc

Approximately 35 mg purified APC5 IgG and Ig^bIgG was digested with 0·35 mg papain (Sigma), so as to give an enzyme: IgG ratio of 1:100, in the presence of Clelands reagent for 45 mins at 37°. The reaction was stopped with 0·006 M iodoacetamide and the fragments purified by elution from an ACA 44 column. Fc and Fab fragments were separated by passage through DEAE Sepharose using 0·01 M HCl Tris buffer, pH 8·5 and a 0–0·2 M NaCl gradient.

Anti-Fdγ, anti-μ, anti-Ig^b and anti-Ig^a

Rat anti-mouse Fdγ was purchased from Sera-lab [Clone YA2/40 H(LK)] and was purified by 50% ammonium sulphate precipitation. Its specificity for Fdγ was demonstrated by the fact that high levels of the radiolabelled antibodies were taken up by IgG and $F(ab')_2$ coated microtitre plates but only low levels by Fcγ- or IgA-coated plates.

Goat anti-μ chain was a gift from Dr D. Dresser (National Institute for Medical Research, Mill Hill). The IgG fraction from this serum was prepared by sodium sulphate precipitation and elution from DEAE Sepharose. Its specificity for mouse IgM was demonstrated by the fact that high levels of the radiolabelled antibodies were taken up by mouse IgM-coated microtitre plates but only low levels by IgG- or IgA-coated plates.

Anti-Ig^b and anti-Ig^a were purified from anti-allotype sera by affinity chromatography as detailed elsewhere (Elson & Taylor, 1974).

Antibody-containing preparations were ^{125}I-labelled using chloramine T.

RESULTS

Production of antibody to host allotype

Groups of mice receiving 10^8 Ig^a-primed spleen cells were left unchallenged or challenged with either one or weekly injections of antibody-coated pertussis. A fourth group was given 10^8 unprimed spleen cells and weekly injections of antibody-coated pertussis. The mice were bled at intervals and their anti-Ig^a allotype antibody response measured. The results (Fig. 1) showed that although anti-Ig^a antibodies appeared in recipients of Ig^a-primed cells a week after cell transfer, the levels declined with time and this was not prevented by repeated challenge with Ig^a-coated pertussis. No anti-Ig^a antibody was detected in recipients of normal cells. The specificity of the anti-Ig^a was tested by assaying positive sera against Ig^b Fc fragment using labelled anti-Fd fragment. No activity was found.

Complexes in immunized mice

We reasoned that the failure to detect serum anti-allotype and antibodies might be because they were circulating in an immune complex. This led us to test the sera for the presence of such complexes. Figure 2 shows the anti-complementary activity of PEG precipitates from serum taken at intervals after spleen cell transfer and during weekly immunization with antibody-coated pertussis. Complement consuming activity appeared 6 weeks after transfer not only of Ig^a-primed Ig^b cells but also of normal Ig^b cells in mice receiving

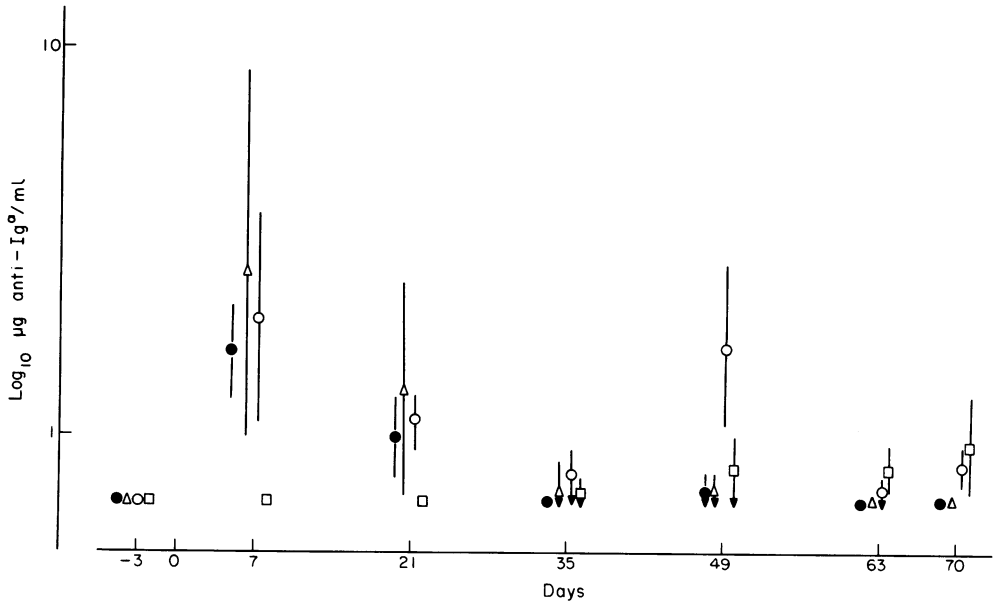

Figure 1. Effect of repeated challenge on anti-host allotype antibody response. Ig^a mice were given: (●) primed Ig^b cells alone; (△) primed Ig^b cells and one injection of Ig^a-coated pertussis; (○) primed Ig^b cells and weekly injections of Ig^a-coated pertussis; or (□) normal Ig^b cells and weekly injections of Ig^a-coated pertussis.

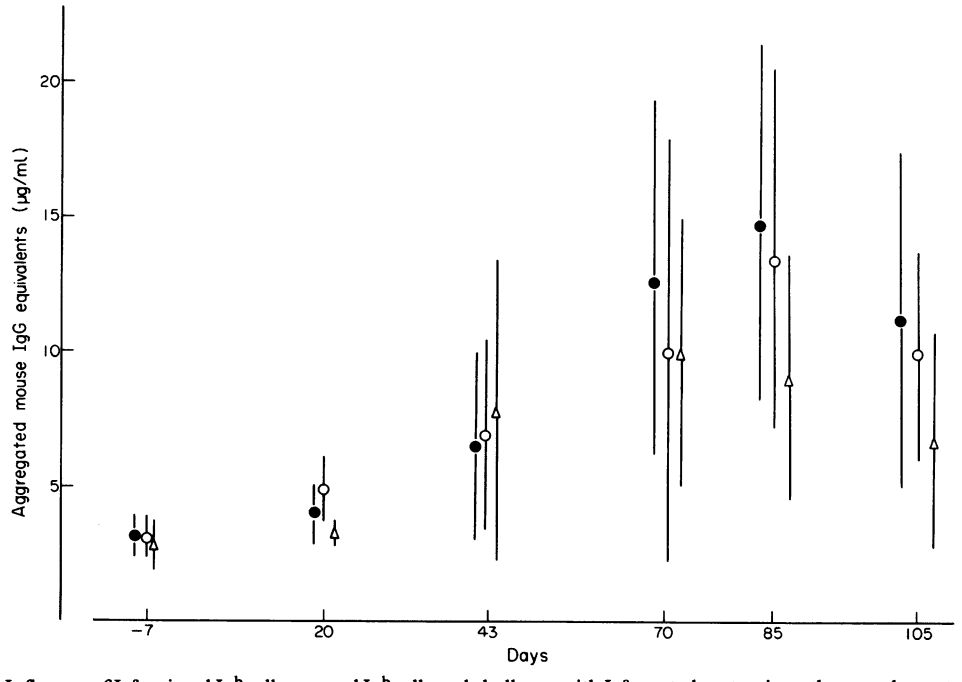

Figure 2. Influence of Ig^a-primed Ig^b cells, normal Ig^b cells and challenge with Ig^a-coated pertussis on the complement consuming activity of serum PEG precipitates. Mice received: (●) normal Ig^b cells; (○) primed Ig^b cells; (△) no cells. They were all injected weekly with Ig^a-coated pertussis.

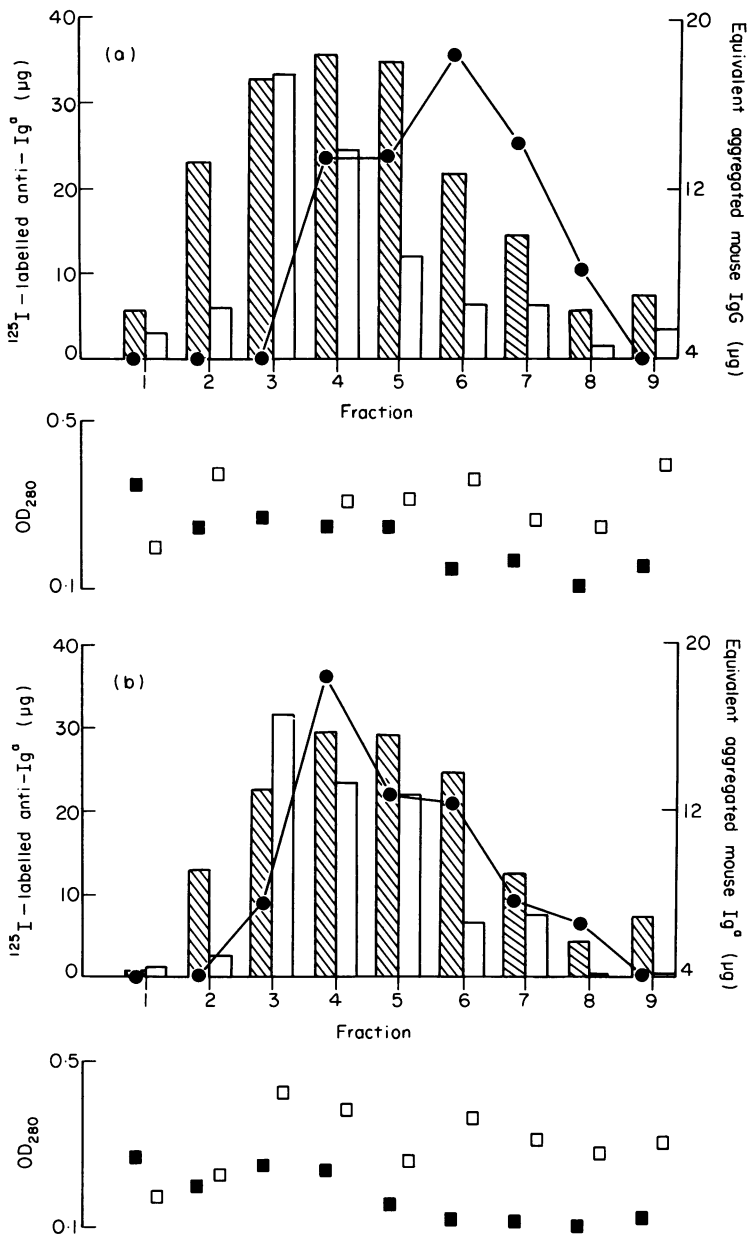

Figure 3. Ig^a allotype, complement consuming activity and protein concentration in CFD and acid sucrose density gradient fractions. (a) PEG precipitates from Ig^a mice which received Ig^b cells and weekly Ig^a-coated pertussis. (b) PEG precipitates from Ig^a mice which received Ig^a-coated pertussis. (▨) CFD gradient; (□) acid gradient.

weekly antibody-coated pertussis. In addition, control mice receiving weekly antibody-coated pertussis alone gave increased complement consuming activity. No activity was detected in mice given cells alone.

Table 1. Anti-Ig^a activity in acid fractions

Treatment	^{125}I-labelled anti-Fdγ bound by fraction (ng)		
	2	3	4
10^8 Ig^b cells day 0 10 weekly Ig^a-coated pertussis injections	58	54	13
10 weekly Ig^a-coated pertussis injections	<0·6	<0·6	0·7
Age-matched controls	<0·6	<0·6	<0·6

Analysis of complexes

It is possible that spleen cell transfer affected the type or constitution of the complexes caused by repeated immunization of antibody-coated pertussis. Accordingly serum PEG precipitates were analysed by sucrose density ultracentrifugation for their Ig^a and anti-Ig^a content. Pools of serum from each of three groups of mice were made. The first pool was from mice which had received Ig^b cells and ten weekly injections of Ig^a antibody-coated pertussis. The second pool was from mice immunized in the same way as group 1 but which had received no Ig^b cells. The third pool was from age-matched normal mice. Mice were bled 7 days after the last injection. PEG precipitates were redissolved in CFD or glycine HCl buffer and ultracentrifuged. First, tests were carried out to confirm that any complexes present had been dissociated by acid treatment. Protein (Fig. 3) was found in all fractions from groups 1 and 2 after CFD sucrose density gradient centrifugation but the amount was increased in fractions 3 and 4 (in which IgG localizes) after ultracentrifugation at acid pH. Complexes were present in the fractions after CFD centrifugation as complement consuming activity was found in fractions 3 to 8 in groups 1 and 2 but none was detected in

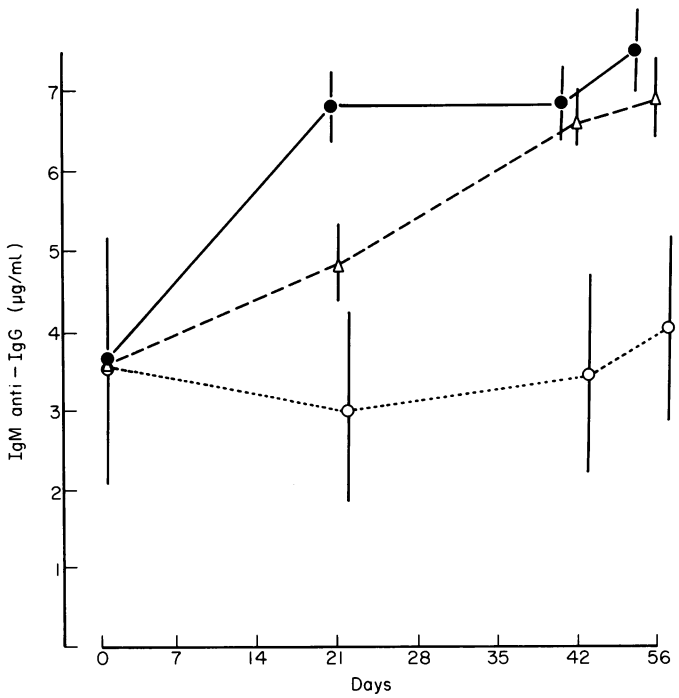

Figure 4. Effect of pertussis on the IgM anti-IgG response. Ig^a mice were injected weekly with (●) Ig^a-coated pertussis, (△) pertussis or (○) left uninjected.

the acid fractions. An estimate of the amount of Ig^aIgG in fractions from groups 1 and 2 is summarized in Fig. 3. It can be seen that after acid centrifugation Ig^aIgG content of fractions 3 and 4 from both groups was increased as compared with fractions 3 and 4 from CFD centrifugation. No Ig^a was detected in fractions from group 3. Tests for the presence of Ig^bIgG in the fractions were negative.

The IgG anti-Ig^a activity of fractions was assayed and found only in acid fractions 2, 3 and 4 from the group 1 pool (Table 1). The fractions were also tested for Ig^b anti-Ig^a activity but none was detected.

Production of IgM rheumatoid factors

Pertussis may act as a polyclonal B-cell activator. As polyclonal activators such as lipopolysaccharide stimulate the production of rheumatoid factors, sera were assayed for their rheumatoid factor activity. Figure 4 shows the rise of serum IgM anti-IgG levels in mice injected with antibody-coated pertussis or pertussis alone. The levels of IgM anti-IgG antibodies in age-matched normal mice did not change over the course of the experiment.

DISCUSSION

The results extend our previous work (Grace *et al.*, 1980) by suggesting that lymphoid cells transferred from allotype congenic donors to recipients may continue to produce anti-host allotype antibodies if appropriately stimulated. Acid dissociation of immune complexes isolated from the sera and synovial fluid of patients with rheumatoid disease has been found to reveal considerably increased titres of rheumatoid factors (Kunkel, Müller-Eberhard, Fudenberg & Tomasi, 1961; Winchester *et al.*, 1970; Jones *et al.*, 1981). Similar treatment of immune complexes from the mice described here revealed anti-host allotype antibodies at a time when they were undetectable in sera. It could be argued that these antibodies were of host origin since antibodies bearing the Ig^b (donor) allotype could not be detected. However, anti-Ig^a activity in the complexes was dependent on the transfer of Ig^b cells to the recipients because complexes from CBA mice given no Ig^b cells, but challenged with Ig^a-coated pertussis, contained no anti-Ig^a activity. The lack of detectable Ig^b anti-Ig^a may merely reflect the fact that less than 20% of antibodies bear the Ig^b allotype (Elson & Taylor, 1974). Ig^a-bearing immunoglobulin was detected in complexes and localized in the 7S fractions after acid dissociation. It seems reasonable to conclude that at least some of this was the antigen with which the anti-Ig^a was combined in the complex. Thus the purpose of this work, to induce a persistent IgG anti-host IgG response and thereby IgG-IgG complexes, was achieved.

The finding of immune complexes in mice immunized weekly with antibody-coated pertussis but given no Ig^b cells was unexpected, particularly as the complex levels remained high for several weeks after the injections were terminated (Grace, unpublished). Usually daily injections of antigen are required to cause the persistence of circulating immune complexes (Steward & Devey, 1981). It is not clear how the injections cause the development of circulating complexes. It has been reported that polyclonal B-cell activators such as bacterial lipopolysaccharide induce IgM rheumatoid factors (Dresser & Popham, 1976; Dresser, 1978). The pertussis injections may act in the same way, as we found a gradual, prolonged increase in serum IgM (but not IgG) rheumatoid factors in these mice, which roughly parallelled the complex levels. Whether these complexes contain IgM rheumatoid factor remains to be determined. In this respect it is of interest that preliminary analysis of the complexes has revealed only a little anti-pertussis activity.

It may be asked if the circulating IgG-anti-IgG complexes had an effect on the mice. So far we have found that more immunoglobulin and C3 was present in the kidneys of the mice as compared with normal animals and also that they are non-specifically immunosuppressed (Grace & Elson, unpublished). However, there were no symptoms of vasculitis or synovitis. There are a number of possible reasons for this. The ability of soluble immune complexes to initiate inflammatory lesions depends on such factors as the amount of antigen-antibody complex present, the affinity of the antibody and the capacity of the complex to activate complement. The amount of complex in the mice may be too low to deposit and induce inflammation or the antibody may be of unsuitable affinity. In addition, although the mouse complexes fix guinea-pig complement it is not known that they fix homologous complement, a capacity which is exhibited by the rheumatoid factor containing complexes found in patients with rheumatoid vasculitis (Elson *et al.*, 1982). Another consideration is that the IgG-anti-IgG complexes in the mice may not induce vasculitis or synovitis in the absence of other abnormalities. This is suggested by the recent

observations of the mouse strains that develop a lupus like syndrome (Steinberg, Huston, Taurog, Cowdrey & Raveche, 1981; Theofilopoulos & Dixon, 1981). It appears that a number of abnormalities, many of which are inherited independently, combine to cause the syndrome. By itself, no one abnormality induces the syndrome. In the same way rheumatoid disease may be caused by the coincidence, in an individual of a number of independent abnormalities. One of these abnormalities may be the production of high levels of IgG rheumatoid factor which if expressed in rheumatoid patients induces a particular symptom or symptoms. Whether the IgG-anti-IgG complexes described here may exacerbate vasculitis or synovitis induced by other means, or if they would cause such pathological conditions when superimposed on an appropriate genetic background, remains a matter for speculation. Nevertheless, the current model may be of use in investigating which factors influence the long term circulation of complexes and if and how complexes effect the immune system.

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