

# IMMUNE COMPLEXES CAN TRIGGER SPECIFIC, T CELL-DEPENDENT, AUTOANTI-IgG ANTIBODY PRODUCTION IN MICE

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Rheumatoid antibodies are autoantibodies specific for the constant portion of IgG. In mice a number of mutant strains produce large quantities of these antibodies (1, 2). Normal mice produce little or no rheumatoid factor (RF)<sup>1</sup> under usual conditions, but can be induced to do so either by injection with B cell mitogens (3, 4) or by multiple immunizations with protein antigens (5). Lipopolysaccharide (LPS), for example, induces a very strong RF response in immunized mice, possibly because these mice contain a high frequency of B cells capable of producing RF. The RF induction observed using multiple antigen administration is probably caused by immune complexes, and data consistent with the hypothesis that immune complex-specific determinants trigger the response have been presented (6).

Recent data (7, 8) showing that immune complexes can act as polyclonal B cell stimulators *in vitro* have raised the possibility that a single mechanism of nonspecific B cell activation may be at work in both immune complex- and LPS-induced RF production. To test this question a protocol was developed to directly stimulate RF production with immune complexes. This has made a direct comparison between the responses to mitogen and immune complexes possible. The results suggest that RF production in autoimmune strains of mice could be caused by many mechanisms.

## Materials and Methods

*Mouse Strains.* A/J, CBA/J, BALB/cJ (+/+ and *nu/nu*), (A/J × BALB/c), and CBA/N mice were 8–14 wk old.

*Immunizations.* In some experiments a double immunization protocol was used for the induction of rheumatoid antibodies. Primary immunizations were done with the protein antigen ovalbumin (OVA) emulsified in an equal volume of complete Freund's adjuvant (CFA). Mice received 0.2 mg of protein emulsion in a total volume of 0.2 ml. For secondary immunizations, 30 µg of protein was suspended in 200 µl saline and injected intraperitoneally. Immune complex immunizations are described in the figure and table legends.

*Antigen Preparation.* *Limulus polyphemus* hemocyanin conjugated with *p*-azophenylar-

<sup>1</sup> *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; CRI, cross-reactive idio-  
type; HGG, human gamma globulin; Lph-Ars, *Limulus polyphemus* hemocyanin conjugated with *p*-azo-  
phenylarsonate; LPS, lipopolysaccharide; MEM, minimal essential medium; OVA, ovalbumin; PBS,  
phosphate-buffered saline; PFC, plaque-forming cell; RF, rheumatoid factor; SRBC, sheep red blood  
cells.

sonate was prepared according to a standard procedure (9). OVA was purchased from Sigma Chemical Co., St. Louis, MO.

*Preparation of Target Erythrocytes.* Ars-coupled sheep red blood cells (SRBC) were prepared as described (10). The final reaction mixture contained 10 mM Ars diazonium salt, 2% (wt/vol) borate-phosphate-buffered saline (PBS), pH 8.2, and 25% (vol/vol) SRBC. After 45 min incubation on ice, the cells were washed sequentially in PBS and minimal essential medium (MEM) (Irvine Scientific, Santa Ana, CA). OVA-coupled cells were prepared by the chromium chloride method (11). Briefly, to an equal volume of packed, saline-washed SRBC was added 1 vol of 10 mg/ml OVA and 1 vol of 2 mg/ml  $\text{CrCl}_3$ . The mixture was incubated 10 min at room temperature and washed again in saline. Coupled RBC were stored in PBS, 1% dextrose at 4°C.

*Monoclonal Antibodies.* id77 ( $G_{2a,\kappa}$ ) and 423E2 ( $G_{1,\kappa}$ ) are anti-Ars-specific proteins from hybridomas isolated in our laboratory. 36-71 ( $G_{1,\kappa}$ ), 31-62 ( $G_{2a,\kappa}$ ), and 45-273 ( $G_{3,\kappa}$ ) are Ars-specific monoclonal antibodies that were kindly provided by Prof. Marshak-Rothstein of Boston University Medical School (12). All of the Ars-specific hybridoma proteins were derived from A/J mice, and all except 423E2 bear the major cross-reactive anti-Ars idiotype (CRI) of A/J mice (13). All of the above-mentioned anti-Ars hybridoma proteins were affinity purified on Lph-Ars- or human gamma globulin (HGG)-Ars-coupled Sepharose 4B. 3A4 ( $G_{1,\kappa}$ ), a CRI<sup>+</sup>, Ars-nonbinding antibody (14), was purified as described (14).

*Plaque Inhibition.* 3A4 protein was used as an inhibitor in some of the plaque assays. Heat-aggregated 3A4 was prepared by incubating a 4 mg/ml solution of 3A4 in PBS for 5 min at 65°C. The resulting suspension appeared opalescent. This preparation was appropriately diluted in MEM and mixed with the other antibody preparations and cells before incubation in the plaque chambers.

*Hemagglutination Assay.* To measure anti-Ig activity of serum samples, hapten-coupled SRBC were sensitized with antihapten antibodies of any of the IgG subclasses. Antibody samples to be tested were serially twofold diluted in saline supplemented with 1% bovine serum albumin, and 50- $\mu$ l aliquots were placed into 96-well microtiter trays. An equal volume of the SRBC solution containing 0.4% hapten-SRBC, 1  $\mu$ g/ml monoclonal anti-hapten antibody, and 1% dextrose was added to each microtiter well. After mixing, the cells were allowed to settle overnight at 4°C. For anti-OVA hemagglutination measurements, the SRBC solution contained no additional antibody and the cells were coupled with OVA.

*Plaque Assay.* We modified the procedure of Cunningham and Szenberg (15) for detecting RF PFC. All reagents and cells were diluted or prepared in serum-free MEM. Equal volumes of 15% (vol/vol) Ars-SRBC, anti-Ars monoclonal antibody at the appropriate dilution, and guinea pig serum (Difco Laboratories, Inc., Detroit, MI; Gibco Laboratories; or Colorado Serum Co., Denver, CO) diluted 1:4 as a source of complement, were added to 1 vol of the antibody-forming cell suspension. This mixture was pipetted into glass slide chambers, sealed, and incubated at 37°C for 35–40 min (longer incubations only slightly increased the maximum number of plaque-forming cells (PFC) detected and often led to a deterioration in plaque quality, making scoring uncertain). Slides were scored for plaques by visualization under a stereoscopic dissecting microscope at 7 $\times$  magnification. 40  $\mu$ g/ml monoclonal antibody was used for most of the assays, with the exception of 36-71, which developed plaques maximally at a concentration of 20  $\mu$ g/ml.

*Preparation of  $F(ab')_2$  Fragments.* 36-71 and id77 were dialyzed against 0.1 M Na acetate, 0.2 M NaCl, pH 4.5 at room temperature. Crystalline porcine pepsin (Worthington Biochemical Corp., Freehold, NJ) was added to each at a final concentration of 100  $\mu$ g/ml and the digestions were allowed to proceed for 18 h at 37°C. The solutions were neutralized by the addition of concentrated Tris-HCl. Undigested material was removed by passage over Sepharose-coupled staphylococcal protein A (Sigma Chemical Co.). Flow-through fractions were concentrated by dialysis against Aquacide IIA (Calbiochem-Behring Corp., La Jolla, CA) and the concentrated material was redialyzed against PBS before use.

*Reduction and Alkylation of IgG.* Antibody at a concentration of 1 mg/ml in PBS was

reduced by the addition of dithiothreitol to a concentration of 10 mM, followed by incubation for 30 min at 37°C. Alkylation was achieved by the addition of 1/11 vol of 0.3 M iodoacetamide in 0.3 M Tris HCl, pH 8.2 and incubation at room temperature in the dark for 20 min. Samples were then dialyzed extensively against PBS before use.

### Results

*Double Immunization Protocol for the Induction of Rheumatoid Antibodies.* It was previously shown (5) that secondary (but not primary) immunization of mice with a variety of soluble protein antigens can result in the production of large amounts of rheumatoid factor-like autoantibodies. Fig. 1 shows that one property of this response is a striking dependence upon antigen dosage. The response peaks at a secondary immunization dose of 11.1  $\mu\text{g}$ , resulting in 38,000 anti-IgG1 PFC/spleen. In the group receiving no secondary immunization, autoantibody production was not observed. In this experiment, A/J mice were immunized with OVA in CFA and then boosted 2 mo later with various amounts of soluble OVA. After 4 d, splenic anti-IgG1 PFC responses were measured.

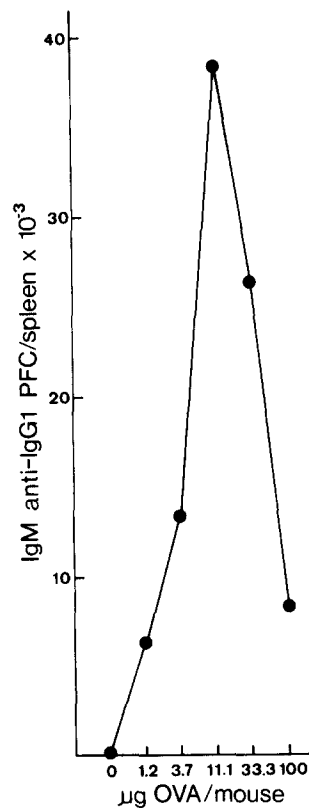


FIGURE 1. OVA-primed A/J mice produced anti-IgG1 in response to secondary stimulation with OVA. Mice were primed by an injection of 200  $\mu\text{g}$  OVA in CFA. After 2 mo, mice were injected intraperitoneally with various amounts of soluble OVA in PBS. Anti-IgG1 splenic PFC were determined after 4 d. Each point represents the mean value determined from a pool of three spleens. Background PFC to the irrelevant antigen, Ars-SRBC, were low (320–1,320 PFC/spleen) in all groups and did not depend on the dosage of OVA.

*Immune Complexes Directly Induce Rheumatoid Antibody Production.* A protocol was developed for induction of rheumatoid antibodies that relies on the direct stimulation of naive animals with exogenously supplied immune complexes. Table I details an initial experiment using anti-Lph-Ars serum as the source of antibody. Mice that were injected with both antiserum and the Lph-Ars protein responded with anti-IgG1 production, detected 4 d later, whereas mice receiving either of these two reagents alone produced very low levels of rheumatoid antibody. The magnitude of the response to IgG1 was large relative to the anti-Ars response measured in the same mice. No anti-Ars PFC response was detectable in the group of mice receiving Lph-Ars alone.

A protocol in which anti-Lph-Ars antibody was delivered intravenously at the same time as an intraperitoneal injection of Lph-Ars was more effective in inducing an anti-IgG1 response than a mixture of the two given intraperitoneally (Table I). In this experiment, as in the one shown in Fig. 1, the ratio of antibody to antigen, rather than absolute amounts of the two, seemed important in eliciting the response (compare lines 1 and 2 in Table I). Anti-Ars PFC were increased in mice injected with anti-Ars serum alone (Table I, group 4) relative to controls that received no antibody. This result was not reproducible using monoclonal anti-Ars antibody (Table II).

*Immune Complexes at Equivalence or Antigen Excess Induce Rheumatoid Antibodies.* To prove directly that it was the anti-Lph-Ars antibody component in the serum that was important in combining with Lph-Ars to induce rheumatoid factor, we substituted syngeneic (Ig4a) affinity-purified monoclonal IgG1 anti-Ars antibody for anti-Lph-Ars serum in subsequent experiments. Table II shows that the monoclonal antibody 36-71 can induce RF-like antibody production in

TABLE I

Group	Anti-Lph-Ars <sup>‡</sup>	Lph-Ars <sup>§</sup>	PFC/spleen*		
			IgM anti-IgG1	Anti-Ars	
				IgM	IgG
	<i>μl</i>	<i>μg</i>			
1	200 i.v.	30 i.p.	11,980	440	360
2	100 i.v.	30 i.p.	20,160	40	0
3	(100 i.p. plus 30 i.p.)		4,000	600	0
4	100 i.v.	—	120	2,280	640
5	—	30 i.p.	120	0	0
6	—	—	680	80	0

Passively administered antibody to Lph-Ars given along with Lph-Ars induces the production of autoanti-IgG1 in A/J mice. In groups 1 and 2, mice were injected first with antibody, then immediately with antigen. Group 3 mice received a single injection containing the antibody and antigen, which had been mixed together within 1 min of injection.

\* Mean of duplicate assays using pooled spleen cells from three mice per group. Background PFC against highly conjugated nitroiodophenol-coupled SRBC did not differ between groups (data not shown).

<sup>‡</sup> The anti-Lph-Ars serum was obtained from A/J mice and contained 0.6  $\mu$ g Ars-binding capacity per microliter as determined by immunoprecipitation.

<sup>§</sup> Lph-Ars was diluted to 150  $\mu$ g/ml in PBS and 0.2 ml/mouse was injected alone (groups 1, 2, and 5) or mixed with serum (group 3).

TABLE II

Group	36-71 (i.v.) <sup>‡</sup>	Lph-Ars (i.p.)	IgM PFC/spleen*	
			Anti-IgG1	Anti-Ars
		$\mu\text{g}$		
1	300	50	4,760	370
2	600	50	7,466 <sup>§</sup>	<40
3	—	50	360	<40
4	600	—	160	<40

Monoclonal IgG1 anti-Ars/Lps-Ars complexes induced anti-IgG1 production in BALB/c mice. Mice were immunized 4 d before sacrifice.

\* See Table I.

<sup>‡</sup> 36-71 was affinity purified over HGG-Ars-coupled Sepharose 4B, eluted with 3 M KSCN, and dialyzed extensively against PBS. The antibody was delivered in a total volume of 200  $\mu\text{l}$  in PBS.

<sup>§</sup> <40 anti-IgG1 PFC/spleen were detected when Ars-SRBC were coated with (Fab')<sub>2</sub>-36-71.

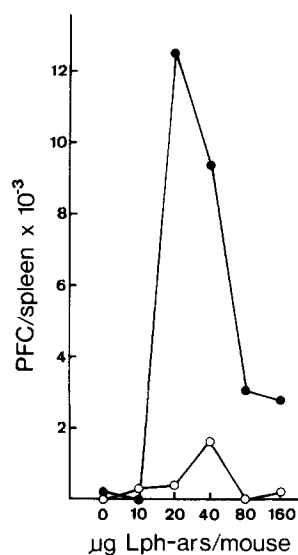


FIGURE 2. Determination of monoclonal IgG1 anti-Ars/Lph-Ars ratio that induces maximal RF production. BALB/c mice (three per group) were injected intraperitoneally with 0.2 ml of PBS containing various amounts of Lph-Ars. In addition, all mice were injected with 600  $\mu\text{g}$  36-71 protein i.v. PFC were determined 4 d after injection. Anti-Ars and anti-SRBC PFC numbered <400 per spleen in all groups (data not shown). IgM anti-IgG1 (●), IgM anti-IgG2a (○).

combination with injected Lph-ars. Neither 36-71 nor Lph-Ars alone had any effect.

Fig. 2 shows the dependence of the autoanti-IgG1 response on the (IgG1) antibody/antigen ratio used to immunize the mice. A pattern similar to that in Fig. 1 was observed. Mice received a fixed amount of 36-71 (IgG1 anti-Ars) intravenously and were subsequently injected intraperitoneally with a solution containing the indicated amounts of Lph-Ars. An antibody/antigen ratio of 15:1

(wt/wt) was found to maximally stimulate production of anti-IgG1. Fig. 3 shows that in quantitative immunoprecipitation studies, using the same 36-71 and Lph-Ars preparations used for the induction of rheumatoid antibodies, equivalence was also reached at a 36-71/Lph-Ars ratio of 15:1.

*Specificity of Immune Complex-induced Antibody.* Fig. 4 shows that anti-IgG1 induced by immunization with immune complexes is specific for the Fc region of the IgG1. When F(ab')<sub>2</sub> IgG1 was used to coat target cells, free PFC were detected. Heat-aggregated IgG1 added at the time of the assay could specifically

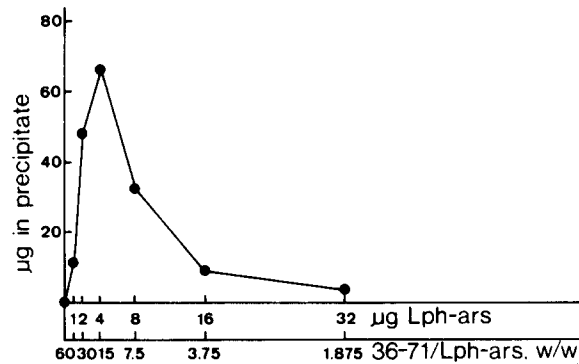


FIGURE 3. Quantitative precipitation of Lph-Ars with monoclonal anti-Ars 36-71. Increasing amounts of antigen were added to 20 µl of 3 mg/ml antibody in a total volume of 52 µl of PBS. After incubation overnight at 4°C, precipitates were centrifuged and washed twice in cold PBS. Protein concentrations were determined by dissolving pellets in 700 µl Lowry C buffer (2% NaCO<sub>3</sub>, 0.1 M NaOH, 0.01% CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.02% Na citrate), incubating 10 min at room temperature, then adding 70 µl of 1 N Folin-Ciocalteu reagent. After 30 min, the absorbance at 750 nm was measured.

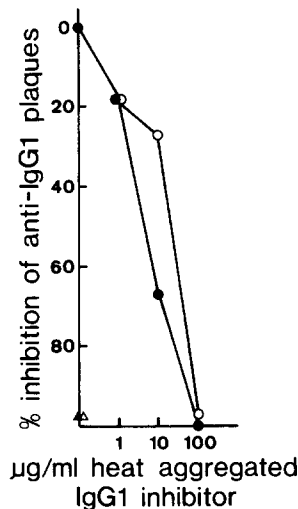


FIGURE 4. Inhibition of anti-IgG1 plaques produced by spleen cells from OVA-hyperimmune (○) or 36-71/Lph-Ars-immunized (●) mice. Various amounts of heat-aggregated (5 min, 65°C treated) IgG1 were added to the indicated final concentrations in the plaquing chambers. (▲, △) Relative amounts of PFC detected using F(ab')<sub>2</sub> instead of IgG1-coated target cells.

inhibit the development of plaques specific for intact IgG1. The concentration of heat-aggregated IgG1 needed to inhibit plaques and the Fc specificity of the PFC were entirely comparable to the anti-IgG1 PFC found in mice after multiple immunizations by protein antigens (5).

*Kinetics of RF Antibody Production in Response to Complexes.* The kinetics of RF PFC production in immune complex-immunized mice were comparable to those of antigen-hyperimmunized mice (Fig. 5). The double immunization protocol results in maximum PFC responses 3–4 d after the second immunization (5), whereas the immune complex immunization resulted in a peak response on day 4 postimmunization. This difference is probably determined by the rate of equilibration of the intravenously injected antibody in the tissues of immune complex-immunized mice.

As in the case of mice hyperimmunized with antigen, only rheumatoid antibody-forming cells of the IgM isotype were detected in these experiments (data not shown).

*CBA/N Mice Respond to Immune Complexes and Produce Rheumatoid Antibody.* When mice bearing the *xid* genetic defect were subjected to the double immunization protocol (see Fig. 1), they were capable of normal RF production relative to congenic control mice (Table III). Mice expressing the *xid* phenotype respond poorly to a variety of T cell-independent (TI-2) antigens (16). These results suggest that the rheumatoid antibody response is clearly not of the TI-2 type and that it requires T cell "help" or is a T cell-independent response of the TI-1 type.

Table IV shows that *xid* mice can respond to immune complexes and produce anti-IgG1. Once again there appeared to be no requirement for antigen-primed lymphocytes for this response. Three of three mice immunized with immune complexes responded by producing large amounts of anti-IgG1, while unimmu-

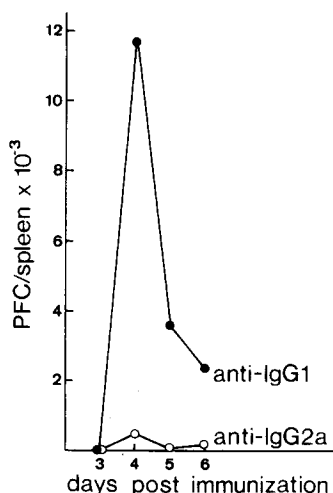


FIGURE 5. Time course of the BALB/c anti-IgG response to immune complex immunization. Mice were injected with 0.6 mg 36-71 (IgG1 anti-Ars) i.v. and 50  $\mu$ g Lph-Ars i.p. on various days before sacrifice. Each point represents the mean value determined from a pool of three spleens. Anti-IgG1 (●), anti-IgG2a (○).

TABLE III

Group*	Anti-IgG1 PFC/spleen		Anti-OVA serum titer	
	CBA	CBA/N	CBA	CBA/N
No immunization	<20	<20	<2	<2
Primed but not boosted	280	50	2 <sup>8</sup>	2 <sup>8</sup>
Primed and boosted	2,640	2,100 <sup>‡</sup>	2 <sup>8</sup>	2 <sup>9</sup>

*xid* mutant mice produce rheumatoid antibodies in response to secondary antigen stimulation. 12-wk-old mice were immunized with 200  $\mu$ g OVA in CFA and boosted 14 d later with 30  $\mu$ g of OVA in PBS.

\* Two mice per group.

<sup>‡</sup> >97% of these PFC were Fc specific as shown by their inability to lyse F(ab')<sub>2</sub>-IgG1-coated SRBC.

TABLE IV

Mouse No.	Treatment	IgM PFC/spleen*	
		Anti-IgG1	Anti-Ars
1	Immune complexes (36-71/Lph-Ars)	14,210	160
2	Immune complexes	10,780	125
3	Immune complexes	9,640	40
4	—	<40	<40
5	—	<40	<40
6	—	<40	<40

*xid* mice made autoanti-IgG1 in response to immune complexes. Mice used were 4-mo-old males of the CBA/N strain.

\* Mean anti-IgG1 PFC/spleen from mice 1-3 was 11,542; of these, <40 were found to react with target cells coated with F(ab')<sub>2</sub>-36-71.

nized mice produced no detectable antibody of this specificity. It appears from this and other experiments that immune complex immunization effectively elicits rheumatoid antibodies from widely different mouse strains. In addition, CBA/N mice share the same IgG1 allotype (IgG4a) as that used for the immune complex immunization, and both the anti-IgG1 from immune complex- and OVA-immunized mice were Fc specific, indicating that the response is truly autospecific (Tables III and IV).

*Failure of Immune Complexes to Induce RF-like Antibodies in Nude Mice.* To determine the cellular requirements of the anti-IgG1 response to immune complexes, we tested the ability of genetically athymic *nu/nu* mice to respond to complexes. Fig. 6 shows that these mice were unable to respond to the complexes by making RF, while control mice consistently responded to stimulation with the complexes. Because *nu/nu* mice are able to mount T cell-independent antibody responses of both the TI-1 and TI-2 types, but cannot mount T cell-dependent responses, this experiment strongly suggests that the rheumatoid antibody response to immune complexes requires thymus-dependent cells.

In contrast to the results using complexes, *nu/nu* mice produced large numbers of anti-IgG1 in response to the mitogen LPS (Table V), demonstrating that they do not lack the B cells needed to produce anti-IgG1 in response to the IgG1/



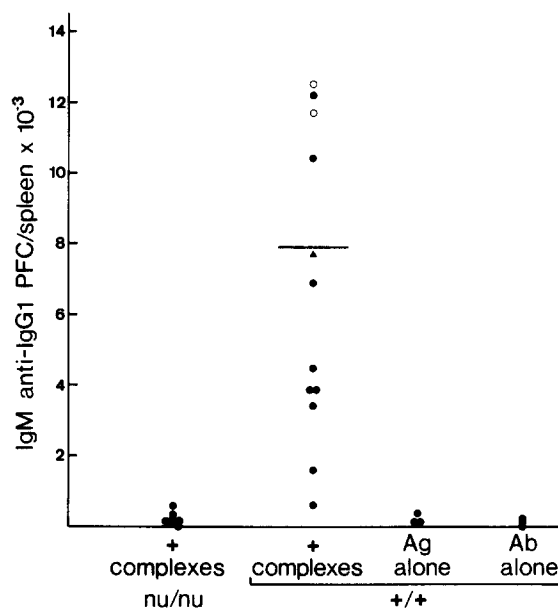


FIGURE 6. Anti-IgG1 PFC production by individual +/+ or *nu/nu* BALB/c mice injected with 0.6 mg 36-71 (i.v.) and 50  $\mu$ g Lph-Ars (i.p.). Splenic PFC were determined 4 d after immunization of animals. Also included are controls in which +/+ BALB/c mice were injected with either 36-71 or Lph-Ars alone. The horizontal bar marks the mean of the indicated values. Individual mice (●), or pools of three mice (○) or four mice (●). This figure combines data from a number of different experiments.

TABLE V

PFC specificity	PFS/spleen			
	<i>nu/nu</i>		+/+	
	LPS	Control	LPS	Control
Anti-SRBC	520	160	1,480	80
Anti-IgG1	8,800	<40	16,120	<40
Anti-IgG2a	720	<40	<40	<40
Anti-IgG2b	1,600	<40	280	<40
Anti-IgG3	200	<40	<40	<40

LPS stimulation of the RF response. Mice received 20  $\mu$ g of *E. coli* LPS intraperitoneally in PBS 4 d before sacrifice. Control mice were injected with PBS alone. Values represent the mean determined from pools of three spleens per group.

antigen complexes. The LPS-inducible levels of anti-IgG1 were comparable in +/+ and *nu/nu* BALB/c mice.

*Immune Complexes Trigger Autoantigen-specific Rheumatoid Antibody Production.* To rule out the possibility that immune complexes themselves could induce polyclonal activation of a type that requires T cells (such as has been described for pokeweed mitogen [17]), I immunized mice with immune complexes made up of Lph-Ars and either IgG1 or IgG2a anti-Ars and examined the specificities of the rheumatoid antibodies produced (Table VI). The clear result is that

TABLE VI

Mouse strain	Group	Monoclonal antibody*		IgM PFC/spleen <sup>†</sup>	
		Anti-Ars	Isotype	Anti-IgG1 (Ig4a) <sup>‡</sup>	Anti-IgG2a (Ig1e)
BALB/c (Iga)	1	id77 (R + A) <sup>‡</sup>	IgG2a	<40	5,960
	2	id77	IgG2a	<40	1,320
	3	36-71	IgG1	7,720	940
	4	423E2	IgG1	5,540	480
(BALB/c × A/J)	5	36-71	IgG1	11,780 <sup>‡</sup>	1,660
(Iga/Ige)	6	id77 (R + A)	IgG2a	<40	7,200**

Stimulation of RF production with various types of immune complexes. Mice were immunized 4 d before sacrifice.

\* All antibody preparations were affinity purified from ascites over HGG-Ars-Sepharose 4B columns. Each mouse received 0.6 mg of antibody i.v., in a total volume of 200  $\mu$ l PBS, along with 50  $\mu$ g Lph-Ars i.p., also in PBS.

<sup>†</sup> Mean values determined from four spleens per group.

<sup>‡</sup> BALB/c mice produced Ig4a and Ig1a; A/J mice produced Ig4a and Ig1e.

<sup>‡</sup> Mice received id77 antibody that was mildly reduced and alkylated to destroy its complement-fixing ability. See Materials and Methods.

<sup>‡</sup> 580 PFC/spleen were observed with F(ab')<sub>2</sub>-36-71-coated target cells (5% of the value with native 36-71-coated cells).

\*\* <40 PFC/spleen were observed with F(ab')<sub>2</sub>-id77-coated target cells.

IgG2a-containing complexes induce the production of anti-IgG2a but not anti-IgG1, and IgG1-containing complexes induce anti-IgG1 production along with much lower levels of anti-IgG2a.

*Effect of Antibody Class and Complement Fixation on the Induction of Rheumatoid Antibodies with Immune Complexes.* One group of mice received mildly reduced and alkylated id77 (IgG2a anti-Ars) along with Lph-Ars. This treatment (18) destroys most of the complement-fixing ability of the antibody but does not affect the overall structure of the antibody or its hemagglutination titer on Ars-coupled RBC (data not shown). As Table VI shows, mice receiving reduced and alkylated IgG2a-containing complexes produced over four times as many anti-IgG2a PFC as those that were injected with normal IgG2a-containing complexes.

A second experiment using (A/J × BALB/c) mice revealed similar results and shows that the anti-IgG2a, much like the anti-IgG1 antibody produced under these conditions, is autospesific and is specific for the Fc portion of IgG (Table VI).

*Comparison of the Specificities of Immune Complex- and LPS-induced Rheumatoid Antibodies.* Both LPS and immune complexes elicit the production of Fc-specific anti-IgG1. Fig. 7 compares the properties of LPS-induced and immune complex-induced anti-IgG1. Spleen cells from mice immunized in these two ways were used in plaque assays in which target cells were coated with either IgG1 or F(ab')<sub>2</sub>-IgG1. No specific PFC were detected in either group using the F(ab')<sub>2</sub>-coated cells. Plaques specific for IgG1 could be inhibited by the addition of heat-aggregated, but not unaggregated, IgG1 in the plaquing chambers. Equal amounts of heat-aggregated IgG1 were needed to inhibit plaques formed by spleen cells from LPS- and immune complex-immunized mice.

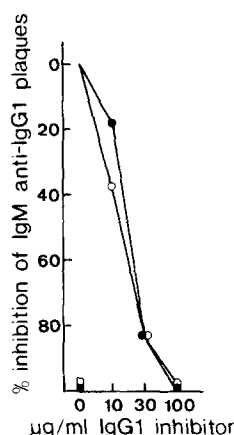


FIGURE 7. Inhibition of anti-IgG1 plaques produced by spleen cells from LPS-treated (open symbols) or immune complex-treated (closed symbols) BALB/c mice. Mice were injected 4 d before sacrifice with 20  $\mu$ g LPS in saline or immune complexes, as described in Table VI. Pooled spleens from three mice per group were used. (O, ●) Degree of inhibition with heat-aggregated IgG1 as competitor. ( $\Delta$ ,  $\blacktriangle$ ) Level of inhibition found when nonaggregated IgG1 was used. ( $\square$ ,  $\blacksquare$ ) Plaques detected with F(ab')<sub>2</sub>-IgG1-coated target cells as a percentage of the values obtained with IgG1-coated cells.

### Discussion

Mice produce rheumatoid autoantibody in response to immune complexes composed of syngeneic antibody and antigen. It has previously been shown (5) that repeated immunization of mice with protein antigens could give rise to RF antibody. In this study, RF antibody production was directly stimulated in previously unimmunized mice by injection of antigen and specific, syngeneic antibody. It was found that the stimulation of RF antibody production by immune complexes in this way was Ig isotype specific: complexes composed of antigen and IgG1 antibody elicited anti-IgG1 while IgG2a-containing complexes elicited anti-IgG2a.

I conclude from these results that the response to immune complexes is not due to polyclonal stimulation of B cells. This result differs from those of Morgan and Weigle (7, 8) that suggest that immune complexes act as polyclonal activators of B cells *in vitro*. In unpublished experiments I have seen no difference between the splenic anti-SRBC and antinitroiodophenyl PFC responses of unimmunized and immune complex-immunized mice, although large RF antibody responses could be demonstrated in the latter, suggesting that nonspecific B cell activation does not occur under the *in vivo* experimental conditions used. I cannot, however, completely rule out the possibility that a relatively small polyclonal stimulation occurs along with a much larger specific anti-IgG response in these mice.

I was interested in assessing the T lymphocyte dependence of the RF response to immune complexes, because the results of earlier experiments involving multiple antigen administration suggested that the anti-IgG response is predominantly of the IgM class and includes little or no IgG antibody. This isotype pattern is typical of T cell-independent responses. The rheumatoid antibody

response to immune complexes appears, in fact, to be T cell-dependent, because it is blocked in T lymphocyte-deficient *nu/nu* mice, but appears to be normal in mice bearing the *xid* mutation. *xid* mice fail to respond to a number of T cell-independent (TI-2) antigens because of the absence of the Lyb-5<sup>+</sup> B cell subpopulation (16).

Although T cells may be required for the RF response, they need not be specifically primed to the antigen or antibody that makes up the immune complex. This result differs from those of Van Snick and Coulie (19) that suggest that spleen cells from mice previously immunized to the foreign parts of the immune complex are required for RF production. The assay system for RF used by these workers may not be sensitive enough to detect the levels of RF produced in response to primary immunization with immune complexes. I believe, however, that antigen-specific T cells can probably augment the response because the levels of RF production achieved with direct immunization of immune complexes never reach those of mice immunized twice with protein antigen following the protocol described.

These data suggest that the previously reported (1) autoreactive anti-IgG2a production of strain 129 mice may be in response to immune complex formation. Consistent with this idea are the elevated IgG2a titers of these mice. NZB, MRL/1, and other strains of mice producing rheumatoid antibody also appear to suffer from immune complex disease and may make the anti-IgG in response to these complexes (2, 20).

Nude mice and normal mice have B cells capable of producing rheumatoid antibody in response to injection with LPS (this study and 21). Because in this study nude mice did not respond to immune complexes, I conclude that two independent pathways of rheumatoid antibody production are possible: (a) T cell-independent, polyclonal B cell activation resulting in production of antibody specific for a wide variety of antigens in addition to self-IgG. (b) T cell-dependent, immune complex-specific, immune responsiveness.

Despite their differing mechanisms of induction and their cellular requirements, LPS- and immune complex-induced rheumatoid antibody have similar binding specificities. Anti-IgG1 produced by spleen cells from LPS- and immune complex-injected BALB/c mice proved indistinguishable from one another in terms of their abilities to bind heat-aggregated IgG1 in a hemolytic plaque assay and in their shared specificities for the Fc portion of IgG, suggesting that a given B cell capable of producing RF could be triggered by two different mechanisms.

The preference for IgG1 in the rheumatoid antibody response to immune complexes caused by hyperimmunization appears to be due to three factors. First, the precursor frequency of B cells with this specificity, as compared with B cells specific for other IgG isotypes, appears to be greater, as judged by LPS-stimulatable anti-IgG splenic PFC. This conclusion is supported by the studies of Van Snick and Coulie (4) of hybridomas derived from LPS-stimulated mouse spleen cells, showing that 58 of 68 autoanti-IgG-specific hybridoma proteins bind IgG1 exclusively. Second, IgG1 is usually produced in somewhat higher concentrations than other IgG classes in a number of antibody responses and so generally makes up a larger fraction of the IgG component of immune complexes in these mice. Finally, the ability of the other IgG isotypes to more efficiently fix

complement may mask the Fc determinants normally seen by the rheumatoid antibody-producing cells, as suggested by the fact that complexes which included non-complement-fixing IgG2a were more effective than complement-fixing IgG2a in eliciting a specific anti-IgG2a response. In addition, immune complexes that have fixed complement are probably cleared more rapidly by phagocytic cells than those that do not. Thus, complement deficiency or complement consumption due to the presence of very large amounts of complement-fixing immune complexes could result in production of rheumatoid antibody specific for the Fc portions of complement-fixing IgG isotypes.

The appearance of roughly equal levels of RF PFC in both nude and normal BALB/c mice injected with LPS suggests that B cells with anti-self antibody specificity are probably not expanded in nonimmune mice, due to ongoing environmental immune complex stimulation. This is because nude mice produce much lower levels of IgG than normal mice and should therefore not be able to develop IgG-containing immune complexes that would stimulate anti-IgG antibody-specific B cells to the same extent as normal mice. These data are supported by Izui et al. (21) who have shown that LPS-induced autoanti-IgG serum levels in *nu/nu* and normal C57BL mice are comparable. These and other studies (22) show that the frequency of B cells capable of producing RF after mitogen stimulation is very high, underscoring the importance of testing the role of polyclonal activation in immune complex-induced RF production.

### Summary

Immunization of mice with a combination of passively administered syngeneic IgG (anti-*p*-azophenylarsonate [anti-Ars]) antibody and a soluble, multivalent form of the antibody's corresponding antigen (*Limulus polyphemus* hemocyanin conjugated with Ars [Lph-Ars]) resulted in specific autoanti-IgG Fc (rheumatoid factor) production. The response was rapid and only anti-IgG of the IgM isotype is found. Because immunization with either the IgG antibody or the antigen alone did not result in rheumatoid antibody production, immune complexes appear to be the active form of the immunogens. Antibody/antigen ratios that resulted in maximal anti-IgG antibody responses were the same as those required for peak *in vitro* immunoprecipitation, i.e., equivalence. Previous exposure of the mice to the exogenously supplied antigen was not required for the response. The response to immune complexes is specific because mice immunized with IgG2a-containing complexes produced autoanti-IgG2a, while mice immunized with IgG1-containing complexes produced anti-IgG1 with little reactivity to other IgG isotypes. IgG2a blocked in its complement-fixing capacity was more effective in eliciting the anti-IgG2a response than native IgG2a, suggesting a possible role for the complement system in modulating the anti-IgG2a response.

Induction of rheumatoid factor production by immune complexes could be induced in *xid* mice but not in *nu/nu* mice, indicating T lymphocyte dependence of the response. In contrast, the B lymphocyte activator lipopolysaccharide was able to elicit vigorous rheumatoid factor production in both *nu/nu* and normal mice, demonstrating that *nu/nu* mice contain B cells capable of making the response. Rheumatoid antibody produced in the immune complex- or LPS-

induced responses is Fc specific and has relatively low affinity for IgG that is not bound to antigen.

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