INDUCTION OF RHEUMATOID ANTIBODIES IN THE MOUSE

Regulated Production of Autoantibody in the Secondary Humoral Response

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Rheumatoid factors (RF) have been classically defined as anti-IgG antibodies, usually of the IgM class, found in the sera of people afflicted with rheumatoid arthritis or a number of other autoimmune diseases (1–3). Antibodies with properties similar to these can be found in mice that display no pathology. We have proposed that such immune complex-specific antibodies be termed "enhancing antibodies" because of their ability to augment antibody:antigen interactions and because we believe that in most cases such antibodies arise normally, produce no concurrent pathogenesis, and may be able to aid in antigen elimination (4). In this scheme rheumatoid factors represent a subset of the enhancing antibodies produced against immune complexes. Others include those that react with new determinants or combinations of determinants generated by the close juxtaposition of antibody and antigen or conformational changes in the antigen due to bound antibody.

In some mouse strains, production of auto-anti-IgG is associated with rheumatoid arthritis- or lupus erythematosis-like syndromes (5, 6). In other strains RF-like material is produced in the absence of obvious disease. Strain 129/Sv mice, for example, appear to produce RF-like antibody in response to some unknown environmental stimulus in an age-dependent manner (7, 8). Mice that do not normally produce autoreactive anti-IgG in detectable amounts can be induced to do so by a number of reagents that are known to be nonspecific B cell activators, e.g. lipopolysaccharide (LPS) (9–12). In this study we have focused on enhancing antibody production and specificity in A/J mice. We show that in mice that do not spontaneously produce detectable amounts of auto-anti-IgG, very large amounts can be induced by repeated immunization with virtually any antigen and the antibody produced is of highly restricted specificity. These results confirm and extend previous work (13–15) that suggested that RF-like antibodies are produced in vivo in response to the presence of immune complexes.

Materials and Methods

Mice and Immunizations. A/J mice were purchased from Jackson Laboratories, Bar Harbor, ME. BALB/c and C57Bl/6 mice were obtained from Charles River Laboratories, Wilmington, MA. (A/J × BALB/c) mice were bred in the Animal Facilities of the Biological Laboratories, Harvard University. In most cases primary immunizations were done with the protein antigen 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.

Antigen Preparation. Ars$_{27}$LPH was prepared according to a standard procedure (16). DNP$_7$KLH was prepared following the method described in (17). OVA was purchased from Sigma Chemical, St. Louis, MO.

Hapten-coupled Erythrocytes. Ars-coupled sheep erythrocytes (SRBC) were prepared as described in (18). The final reaction mixture contains 20 mM Ars diazonium salt, 2% borate (wt/vol)-PBS, pH 8.2, and 25% (vol/vol) SRBC. After a 45-min incubation on ice the cells are washed in PBS. NIP-coupling was done as described in (19). 1 mg of NIP-O-succinamide (Biosearch, San Rafael, CA) is added per 1 ml 20% (vol/vol) SRBC in borate-PBS, pH 8.2 and incubated for 10 min at room temperature. OVA-coupled cells were prepared following the chromium chloride method (20). Briefly, to an equal volume of packed, saline-washed SRBC is added one volume of 10 mg/ml OVA and one volume of 2 mg/ml CrCl$_3$. The mixture is incubated 10 min at room temperature and washed in saline. Coupled erythrocytes were stored in phosphate-buffered saline, 1% dextrose at 4°C.

Monoclonal Anti-hapten Antibodies. id77 (γ_{2a},K) and 423E2 (γ_1,K) are anti-Ars-specific proteins from hybridomas isolated in our laboratory. 36–71 (γ_1,K), 31–62 (γ_{2a},K), and 45–278 (γ_3,K) are Ars-specific monoclonal antibodies that were kindly provided by Professor Marshak-Rothstein of Boston University Medical School (21). 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 (22). D6.582 (γ_1,K) is an anti-NIP monoclonal protein derived from a C57Bl/6 (Ig^b) mouse spleen cell. It was generously provided by Professor Imanishi-Kari of MIT. All of the above-mentioned anti-Ars hybridoma proteins were affinity-purified on LPH-Ars-Sepharose 4B. The anti-NIP monoclonal was affinity-purified on OVA-NIP-Sepharose 4B.

Monoclonal Anti-IgG1 Antibodies. Four rat anti-IgG1 antibodies with RF-like activity, designated 22C9, 24C5, 24D3, and 25C11, have been described elsewhere (4). The hybridoma 411H (γ_3,K) was derived from the fusion of SP 2/0-Ag-14 myeloma cells (23) with the spleen cells of an A/J mouse hyperimmunized with LPH-Ars. G (μ,?) and R (μ,?) were produced by the fusion of the myeloma cells with the spleen cells of KLH-penicillin hyperimmunized Balb/c mice. G, R, P, and Y were produced in collaboration with Angenics, Inc. (Cambridge, MA). All monoclonal antibodies were detected by their ability to bind to antigen:antibody complexes. In the case of 411H, ^{125}I-labeled LPH-Ars:anti-Ars complexes were used; for R, G, Y, and P, ^{125}I-BSA:anti-BSA complexes were used. Hybridoma culture supernatants were applied to polyvinyl chloride wells coated with 50 µl/well specific rabbit anti-mouse Ig as described in (4). After a 2–4-h incubation, anti-mouse Ig sites were blocked by incubation with 1% normal mouse serum for 1 h and then 20,000 cpm of labeled complexes were applied per well in 50 µl of saline supplemented with 1 mg/ml BSA and 1% normal mouse serum. After 2 h the wells were washed extensively in water, cut apart, and the bound radioactivity was measured.

Hemagglutination Assay. To measure anti-Ig activity of serum samples, hapten-coupled SRBC were sensitized with anti-hapten antibodies of any of the IgG isotypes. Antibody samples to be tested were serially twofold diluted in saline-1% BSA and 50-µl aliquots were placed into 96-well microtiter trays. An equal volume of the SRBC solution containing 0.4% hapten-SRBC, 1 µg/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 OVA-coupled. All antisera were heat inactivated

(56°, 30 min) before use.

Plaque Assay. We modified the procedure of Cunningham (24) for use in detecting anti-IgG PFC. Equal volumes of 15% (vol/vol) hapten-SRBC, diluted anti-hapten monoclonal antibody, and fourfold diluted guinea pig serum as a source of complement, were added to one volume of the antibody-forming cell suspension to be tested. This mixture was pipetted into glass slide chambers, sealed, and incubated at 37° for 30–35 min. Slides were scored for plaques by visualization under a stereoscopic dissecting microscope at 7× magnification. 40 μg/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 μg/ml. In some experiments mildly reduced and alkylated anti-hapten antibody was used. Antibodies (1 mg/ml in PBS) were reduced by addition of dithiothreitol to 10 mM and incubated for 30 min at 37°C. Alkylation was carried out on these samples by addition of 10% molar excess of iodoacetamide.

Results

Plaque Assay for RF-like Enhancing Antibodies. We developed a hemolytic plaque assay that detects anti-IgG producing cells with specificity for any of the mouse IgG isotypes. To an aliquot of hapten-modified target erythrocytes is added an appropriate concentration of a monoclonal antibody with specificity for the hapten. The isotype of the antibody can be varied by choosing from a panel of anti-hapten antibodies of different isotypes. The target cells are thus effectively coated with antibody of defined isotype. To this mixture is added antibody-forming cells to be tested and guinea pig complement. The rest of the procedure is essentially identical to the slide method of Cunningham (24). Plaques can be scored after a 30-min incubation at 37°C. In the experiments described here the haptens chosen to modify erythrocytes (Ars, NIP) were unrelated to the antigens used to immunize mice, thus the background levels of plaques detected in the absence of anti-hapten antibody were small.

We have found that the concentrations of antibodies, used to coat cells, which allow maximal plaque detection are sufficiently low that target cell aggregation is not a problem. Complement fixing monoclonals, i.e. IgG2a, IgG2b, and IgG3, must be mildly reduced and alkylated before cell coating to prevent spontaneous target cell lysis. This treatment destroys the complement-fixing ability of the antibody, but appears not to affect RF binding (25, 26). As in standard hemolytic plaque assays, only IgM producing cells are visualized in the absence of "developing" anti-mouse Ig antiserum. To detect IgG3 anti-IgG1 producing cells, for example, it is necessary to add rabbit anti-Ig3 serum to the assay mixture.

Fig. 1 illustrates the dependence of anti-IgG plaque detection on the concentration of monoclonal IgG used to coat the SRBC. In this experiment, spleen cells from either hyperimmune A/J to hyperimmune 129/J mice were used as sources of anti-IgG-producing cells. (Comparisons of the anti-IgG responses of these two mouse strains is the subject of a separate report [Nemazee and Sato, manuscript in preparation].) The fact that the number of plaques detected does not drop in the presence of very large amounts of antibody suggests that most autoanti-IgG plaque-forming cells produce antibody that preferentially binds IgG that is bound to the cells. The inhibition experiment shown in Fig. 4 confirms this point. Also shown in Fig. 1 is the result that mild reduction and alkylation of IgG1 used to coat cells does not effect the number of anti-IgG1 PFC detected by this method. Thus it appears that in A/J mice that have been
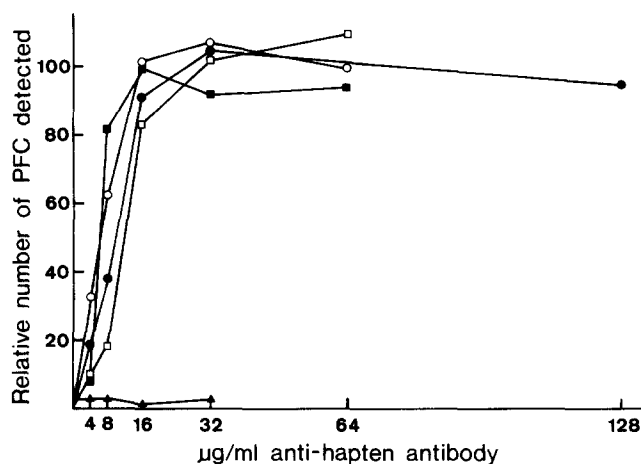
FIGURE 1. Anti-IgG plaques detected as a function of IgG used to coat erythrocytes. Immunoglobulin used to coat cells: IgG4^a (O), reduced and alkylated Ig4^a (●), F(ab)$'_2$-Ig4^a (▲), Ig4^b (□), IgG2a (■). Spleen cells producing anti-IgG1 were obtained from A/J mice (*vide infra*). Cells producing anti-IgG2a were from 129/J mice (7).

hyperimmunized the bulk of the anti-IgG1 producing cells cannot distinguish between native and reduced-and-alkylated IgG.

Induction of RF PFC with Secondary Antigen Stimulation.   Unimmunized A/J mice normally produce little or no autoanti-IgG as detected by hemagglutination, plaque assay, and radioassay (this study and reference 27). We have never observed significant numbers of anti-IgG PFC in mice of this strain of any age. We suspected that hyperimmunization with an antigen could induce production of RF-like material in these mice. Therefore we looked for anti-IgG PFC in the spleens of A/J mice at various times subsequent to primary or secondary immunization with a variety of protein antigens. No significant anti-IgG response was observed in mice injected once with either OVA or DNP$_7$KLH in CFA, soluble DNP$_7$KLH, or CFA alone. However, secondary stimulation with DNP$_7$KLH in primed animals yielded massive and rapid IgM anti-IgG1 PFC responses that peaked on day 3 (Fig. 2). We detected little or no autoanti-IgG2a, -IgG2b, or -IgG3 in these animals. In these experiments anti-IgG producing PFC of only the IgM class were monitored. The anti-DNP PFC responses of these mice were large and included large amounts of IgG1, IgG2a, and IgG2b class PFC (Fig. 3). It appears clear from this experiment that a very large fraction of the total number of antibody forming cells of these mice is producing autoanti-IgG1 3 d after secondary antigen stimulation.

Sera from the mice shown to produce large numbers of autoanti-IgG1 PFC were tested for the ability to agglutinate IgG1-coated erythrocytes. Fig. 1 shows that the peak of the agglutinating response occurs a few days after the peak of the PFC response. (The same is true of the anti-DNP hemagglutinating response [Fig. 3].) Using a monoclonal rat IgM anti-IgG1 standard we have estimated that the peak anti-IgG1 titers represent a concentration of ~25–100 μg/ml RF. Table I shows that sera, derived from test animals, containing the maximum amount of anti-IgG1 hemagglutinating activity contain no anti-IgG2a activity,
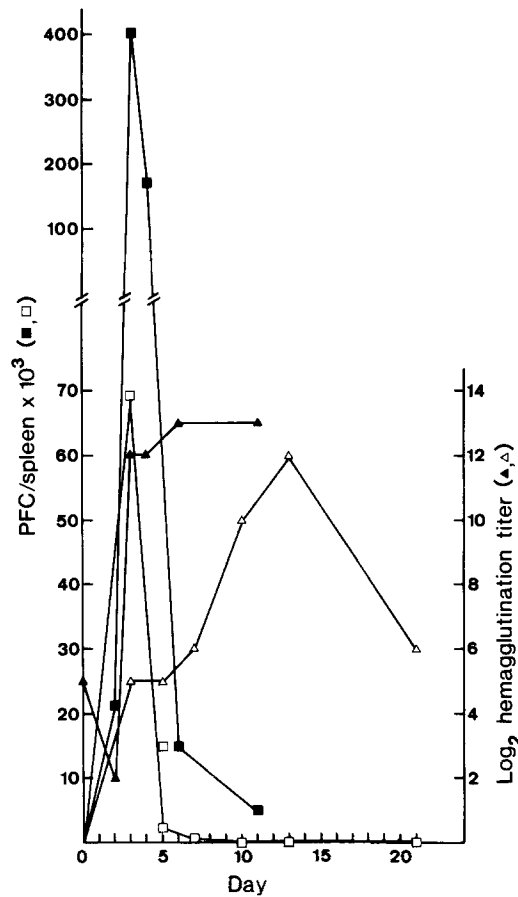
FIGURE 2.   Kinetics of production of anti-IgG1 PFC and hemagglutinating activity in A/J mice following secondary immunization with DNP7KLH. Mice were given a primary dose of 200 μg DNP7KLH in CFA 2 mo before a boosting injection of 30 μg in PBS. The results from two separate experiments are shown: experiment 1 (open symbols), experiment 2 (closed symbols). PFC responses (squares), serum anti-IgG1 titers (triangles). Each point represents the mean value from three mice.

thus confirming our plaque assay results.

The fact that anti-IgG is not induced upon primary immunization suggests strongly that DNP7KLH is not behaving like a polyclonal B cell activator. Such compounds induce RF production after a single injection. Most likely IgG1:antigen complexes stimulate the production of the enhancing antibody. These would not be present after a single injection since the early antibody response to DNP7KLH is predominantly IgM (data not shown), however they would appear upon secondary antigenic exposure.

In order to confirm that DNP7KLH does not induce autoanti-IgG production directly we immunized A/J mice with either DNP7KLH or OVA and boosted the mice some weeks later with one of these two antigens. Splenic E-PFC, specific for IgG1, were measured 3 d after the booster injections. Table II shows that only mice that were boosted with the same antigen as that used for the initial
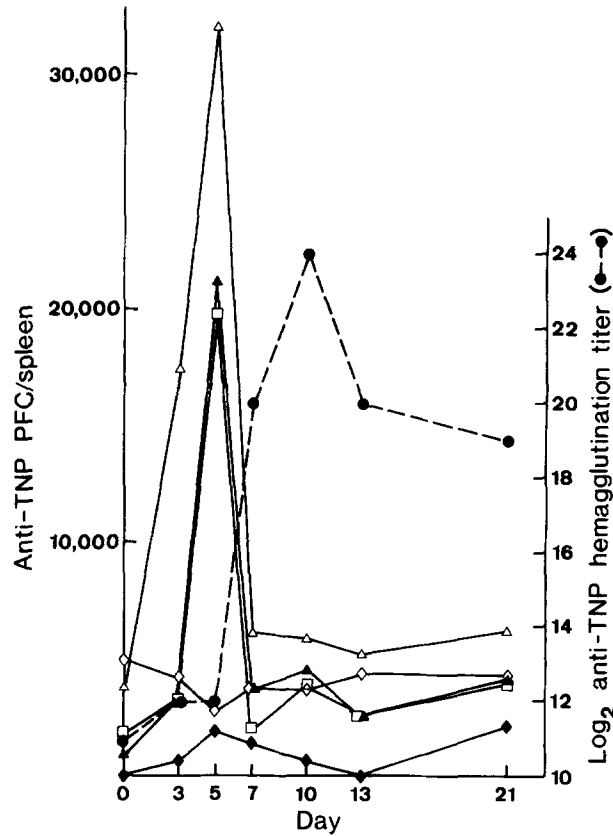
FIGURE 3.   Mice producing IgM anti-IgG1 after secondary immunization to DNP-KLH make a predominantly IgG response to DNP. Spleen cells tested for anti-IgG1 PFC (Fig. 2, experiment 1) were assayed for levels of anti-DNP PFC of various isotypes: IgG1 (△), IgG2a (▲), IgG2b (□), IgG3 (◆), IgM (◇). Sera from these mice were taken and tested for anti-DNP hemagglutinating activity (●).

TABLE I

Anti-IgG1 Hemagglutinating Activity in Twice-immunized A/J Mice

Days post boost*	Hemagglutination titer vs. cells coated with:		
	IgG1 (Ig4ᵃ)	(Ig4ᵇ)	IgG2a
0	<2	<2	<2
13	2,048	512	<2
100 μg/ml 22C9‡	4,096	4,096	0

* Mice were immunized intraperitoneally with 200 μg of DNP₇KLH in CFA. After 2 mo, mice were boosted with 30 μg DNP₇KLH in saline. Groups of three mice were bled and serum collected on various days after the boosting injection.

‡ 22C9 is the designation of a monoclonal rat IgM anti-IgG1 antibody. The antibody was purified from ascites by precipitation in 50% saturated ammonium sulphate and precipitation in 20 mM Na phosphate, pH 7.5.

immunization produced detectable anti-IgG1. Similar results were obtained with C57B1/6, BALB/c, and (A/J × BALB/c)F1 mice (Table II).

Specificity of Anti-IgG1 Antibody. Plaque inhibition experiments were performed to further define the specificity of the enhancing antibody (Fig. 4). In these experiments, A/J mice were induced to produce IgM anti-IgG1 by two rounds of immunizations with OVA. Heat aggregated IgG1 could completely inhibit anti-IgG1 plaques, whereas unaggregated IgG1 could not. Detection of background plaques specific for the hapten NIP were unaffected by heat aggregated IgG1. Isolated Fc and Fab fragments of IgG1 also failed to inhibit anti-IgG1 plaques (not shown). These results suggest that anti-IgG1 antibodies recognize either conformational changes in IgG1 caused by heat aggregation and antigen binding or, perhaps more likely, recognize native IgG1 with low affinity such that clustering of IgG1 molecules is required for high avidity binding.

Twice-immunized A/J mice produce at least two different specificities of autoantibody, one that recognizes both mouse IgG1 allotypes ($Ig4^a$ and $Ig4^b$) and another that recognizes only $Ig4^a$. Plaques of both specificities could be inhibited entirely by the presence of 100 μg/ml heat-aggregated $Ig4^a$ antibody in the plaque assay chamber (Fig. 4). An independent confirmation of these results is detailed in Table I, which shows that hyperimmune A/J serum contains a higher level of anti-$Ig4^a$ hemagglutinating activity than anti-$Ig4^b$ activity. We consistently found that in spleens from $Ig4^a$ strain mice producing anti-IgG1, anti-$Ig4^b$ PFC levels were lower than anti-$Ig4^a$ PFC levels (Table II). The reverse was true in one set of $Ig4^b$ mice tested.

It has been noted that probably a very large fraction of B cells in both mice (9) and humans (28) are specific for self-Ig. Therefore one interpretation of our results indicating that secondary immunization triggers autoanti-IgG production is that the boosting antigen causes production of antibody that cross-reacts between the foreign antigen and autologous IgG. This rather remote possibility is clearly ruled out by the results shown in Fig. 4. Detection of anti-IgG PFC

TABLE II
Anti-IgG1 is Produced in Response to Secondary Antigenic Stimulation

Strain	Priming Ag*	Boosting Ag	IgM anti-IgG1 Anti-Ig4*	PFC/ spleen‡ Anti-Ig4‡
A/J	OVA	OVA	130,606	72,618
	OVA	DNP-KLH	1,000	ND
	DNP-KLH	OVA	533	1,066
	DNP-KLH	DNP-KLH	57,600	ND
BALB/c	OVA	OVA	36,800	23,200
	DNP-KLH	OVA	800	0
C57B1/6	OVA	OVA	22,400	24,000
	DNP-KLH	OVA	2,527	8,114
(BALB/c × A/J)F1	OVA	OVA	92,800	45,600
	DNP-KLH	OVA	589	0

* Mice were immunized intraperitoneally with 0.2 mg of either DNP_7KLH or OVA in CFA, rested for 2 mo, and boosted 3 d before the assay with 30 μg of either antigen in saline.
‡ Assays were done 3 d after the second injection. Each group consisted of three mice.
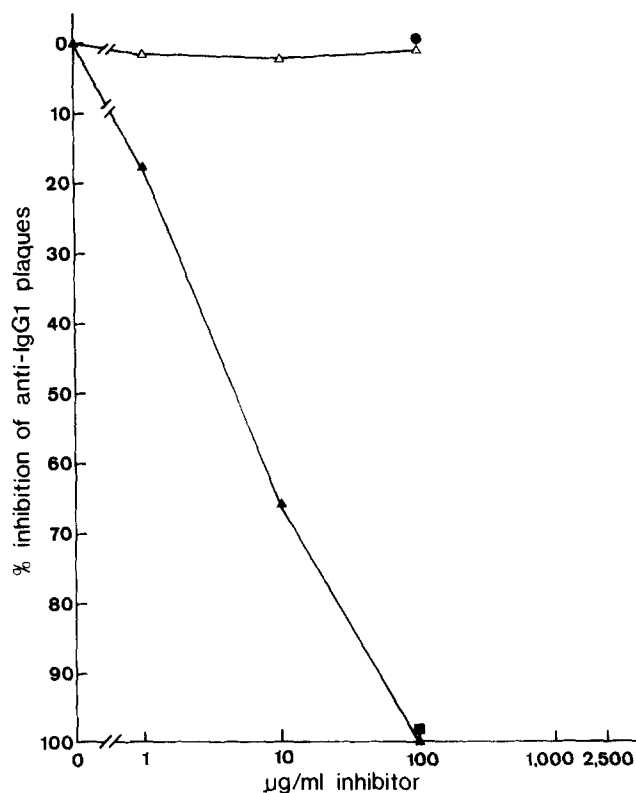*ND*, not determined.

FIGURE 4. Inhibition of A/J IgM anti-IgG1 plaques. Spleen cells pooled from three A/J mice twice immunized with OVA were used as a source of anti-IgG1 producing cells. Inhibition of anti-Ig4ᵃ plaques by: unaggregated Ig4ᵃ (△), heat aggregated Ig4ᵃ (▲), OVA (*). Inhibition by heat aggregated Ig4ᵃ of anti-Ig4ᵇ plaques (■), anti-NIP plaques (●).

from the spleens of mice immunized twice with OVA was unaffected by the presence of 2.5 mg/ml OVA in the plaquing chamber.

We obtained several monoclonal autoanti-IgG1 producing cell lines, derived from both mouse and rat, using somatic cell fusion techniques. Table III shows that of five monoclonal anti-IgG1 antibodies derived from Igᵃ-strain mice, four recognize Ig4ᵃ exclusively and one recognizes both Ig4ᵃ and Ig4ᵇ. As yet we have not found a hybridoma that produces an antibody specific for any of the other mouse immunoglobulin isotypes. None of the rat antibodies tested could distinguish between mouse IgG1 allotypes.

Portion of the IgG1 Molecule Recognized by anti-IgG1. We were able to localize the region of the IgG1 molecule that is bound by autoantibody from hyperimmunized mice to the Fc region. Three pieces of evidence support this conclusion. First, F(ab)′₂ IgG1-coated erythrocytes were not agglutinated by a hyperimmune serum pool from A/J mice that had high titer against IgG1-coated cells (Table III). Second, none of the five mouse monoclonal anti-IgG1 antibodies was capable of agglutinating F(ab)′₂ coated erythrocytes (Table III). Finally, in spleens in which very large numbers of anti-IgG1 antibody-forming cells were detected using IgG1-coated cells, no cells were found that produced antibody against

TABLE III

Isotype Specificity of Polyclonal and Monoclonal RF

| Name | Source | Type | Hemagglutination titer* vs. cells coated with: | | | | | |
| | | | IgG1 | | | IgG2a | IgG2b | IgG3 |
			Ig4[a]	Ig4[b]	F(ab)'$_2$ −Ig4[a]			
411H12	A/J	IgG3	2^{13}	0	0	0	0	0
Y	BALB/c	IgM	$>2^{12}$	0	0	0	0	0
P	BALB/c	IgM	$>2^{12}$	0	0	0	0	0
G	BALB/c	IgM	$>2^{12}$	$>2^{12}$	0	0	0	0
R	BALB/c	IgM	$>2^{12}$	0	0	0	0	0
22C9	CD rat	IgM	2^{15}	2^{15}	0	0	0	0
24C5	CD rat	IgM	$>2^{12}$	$>2^{12}$	0	0	0	0
24D3	CD rat	IgM	$>2^{12}$	$>2^{12}$	0	0	0	0
25C11	CD rat	IgM	$>2^{12}$	$>2^{12}$	0	0	0	0
A/J	Normal serum		<2	ND	<2	<2	<2	<2
A/J	Hyperimmune serum		2^8	ND	<2	<2	<2	<2

* Titer of monoclonal antibodies assumes starting concentration of 1 mg/ml pure protein.
ND, not determined.

F(ab)'$_2$-coated cells (Fig. 1). This result indicates that anti-antibodies of the type described by Milgrom and associates (29, 30), which are found in some human sera and bind to F(ab)'$_2$ fragments that are bound to antigen, are not produced to any great extent in hyperimmunized mice.

Isotype of Anti-IgG1 Material. We found that the predominant isotype of autoanti-IgG1 was IgM. We determined this by using the plaque assay we developed to look for anti-IgG1 producing cells of different isotypes in the spleens of A/J mice. We were fortunate to have available hybridomas producing monoclonal anti-IgG1 of either the IgM or IgG3 class to determine optimal conditions for development of anti-IgG1 plaques. We found that IgM anti-IgG1 plaques form without additional "developing" antisera. Indeed anti-μ sera inhibit these plaques to the same extent as they inhibit IgM anti-erythrocyte plaques. IgG3 anti-IgG1 plaques could be visualized with the addition of anti-γ-$_3$-specific antiserum at a concentration that is also optimal for the detection of IgG3 anti-erythrocyte plaques. Thus standard developing antisera used for hemolytic plaque assays need not be retitrated for use in detection of RF PFC. Using these conditions we could find no evidence for the production of IgG2a-, IgG2b-, or IgG3-anti-IgG1 antibodies in unimmunized or hyperimmunized A/J mice, including mice that were immunized repeatedly with antigen (data not shown).

Clearly some IgG3 anti-IgG1 may be produced since we obtained a hybridoma line derived from a hyperimmune A/J mouse that produces antibody of this kind (Table III). However, the magnitude of the IgG3 anti-IgG1 response in hyperimmune mice must be small relative to the IgM anti-IgG1 response. It should be noted that assay conditions that allow detection of IgG3 anti-IgG1 PFC also reveal all IgM anti-IgG1 PFC. Therefore, in order for the IgG3 PFC to be reliably detected they must be present in numbers not less than about 10% of the number of IgM anti-IgG1 PFC.

In one experiment (Fig. 5) in which an accelerated immunization schedule was
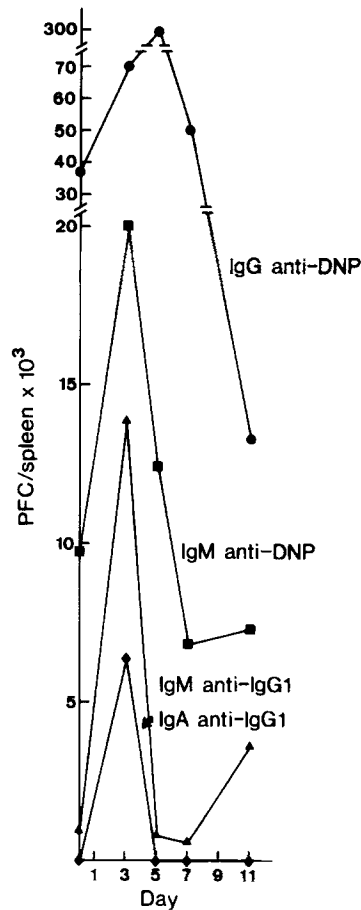
FIGURE 5.   Production of IgM anti-IgG1 in A/J mice twice immunized with DNP₈OVA. Mice were immunized first with 100 μg of protein in CFA and then boosted 10 d later with 30 μg of the protein in saline. Shown are anti-DNP and anti-IgG1 PFC responses various days after the second injection. IgG anti-DNP (●), IgM anti-DNP (■), IgM anti-IgG1 (▲), IgA anti-IgG1 (◆). Each time point represents the mean determination of duplicate samples taken from a pool of three spleens.

used, we detected a significant number (~10,000) of IgA anti-IgG1 PFC in the spleens of A/J mice 3 d after secondary antigenic stimulation with DNP₈OVA, however we were unable to ascertain whether IgA anti-IgG1 hemagglutinating material was present in the sera of the mice. In a number of other experiments using OVA as the antigen we found no difference in the number of anti-IgG1 PFC detected in the presence or absence of anti-α serum.

To confirm our plaque assay results indicating that the bulk of the anti-IgG1 produced in hyperimmunized mice is of the IgM class, we analyzed the physiochemical properties of the anti-IgG1 hemagglutinating activity of hyperimmune sera. Fig. 6 shows that >95% of the hemagglutinating activity of pooled sera from A/J mice immunized three times with ovalbumin is excluded by Sephadex G-200 indicating the material has a molecular weight in excess of 500,000
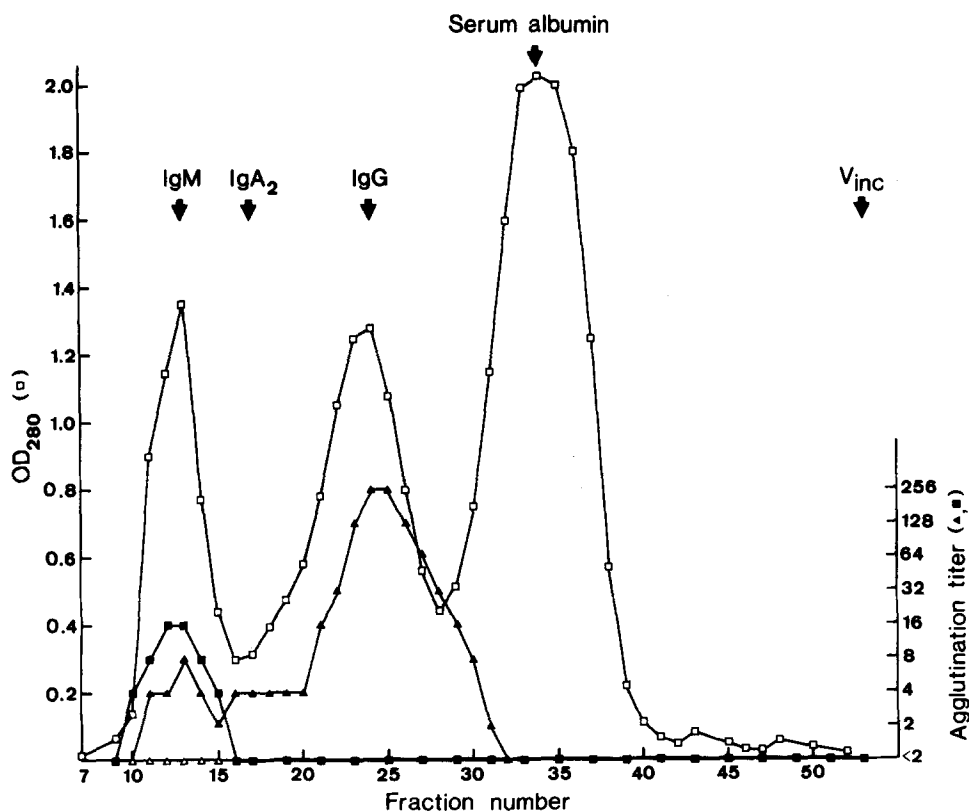
FIGURE 6. Sephadex G-200 gel filtration of A/J anti-OVA serum. One ml of serum was applied to a 50 cm × 1.2 cm column equilibrated with PBS. Anti-IgG1 titers were determined as described in Materials and Methods. Anti-OVA titers were determined by hemagglutination of OVA-coupled sheep erythrocytes. Mice were immunized with 200 μg OVA on CFA on day 0, and boosted with 30 μg in saline on day 10. Serum was harvested and pooled from five mice 7 d after the second injection. Optical density at 280 nm (□), anti-IgG1 agglutination (■), anti-OVA agglutination (▲), anti-F(ab′)2 IgG1 agglutination (△).

daltons. Most of the anti-OVA activity appears to be IgG, running at an apparent molecular weight of <200,000 daltons. The result of DEAE-cellulose chromatography of hyperimmune serum is shown in Fig. 7. Anti-IgG1 activity is eluted at a relatively high salt concentration characteristic of IgM (150 mM), whereas the anti-OVA material is eluted at a much lower salt concentration (50–100 mM), typical of IgG.

Discussion

We have presented evidence that strongly suggests that immune complexes consisting of circulating IgG and injected antigen can induce in vivo production of autoanti-IgG1. We wish to view the RF-like antibodies produced using this system in terms of the enhancer antibody concept, that is, as molecules whose production is induced by immunogenic determinants of immune complexes and whose preferential specificity is for immune complexes. Although self molecules are recognized by the RF-like antibodies, no breakdown of self tolerance is
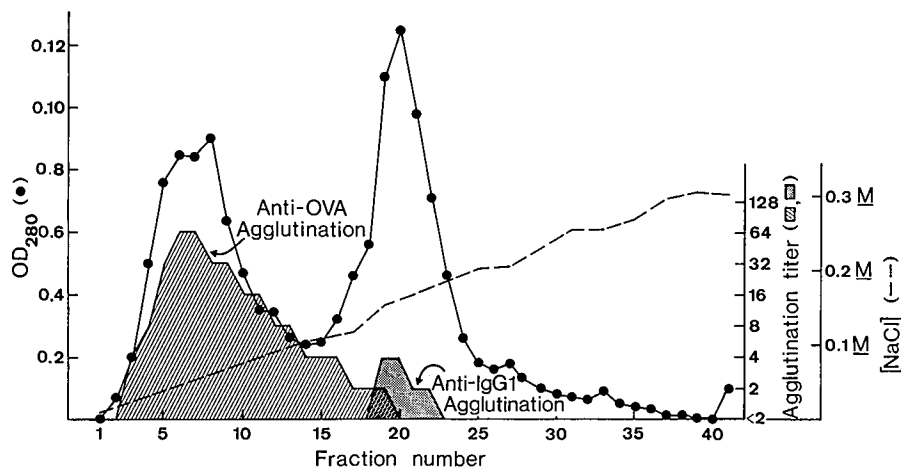
FIGURE 7.   DEAE-cellulose ion exchange chromatography of hyperimmune A/J anti-OVA serum. 50 μl of serum was diluted in 2 ml of 10 mM Na phosphate buffer, pH 7.5 and applied to a 1-ml column equilibrated in the same buffer. Protein was eluted with a linear gradient of 0–0.3 M NaCl in 10 mM Na phosphate, pH 7.5. A group of five mice was immunized with 200, 30, and 100 μg OVA 25, 18, and 7 d, respectively, before serum was taken. For the first injection the antigen was emulsified in CFA, the antigen was diluted in saline for the next two injections.

necessarily implied (Fig. 4), and the response is terminated when the antigen is cleared, underscoring the important contribution of foreign matter in the response. This work shows that secondary immunization of mice with a wide variety of protein antigens can result in autoanti-IgG production. Similar experiments by others (31, 32) using rabbits hyperimmunized to whole bacteria have yielded similar results. We have extended this analysis by assessing the isotype and allotype specificities of RF produced in hyperimmunized animals. The striking result is that the range of anti-IgG specificities expressed is very limited. In A/J mice virtually all of the RF produced is IgM anti-IgG1 Fc.

We have developed a modified hemolytic plaque assay to detect antibody forming cells that is similar in design to RF-specific plaque assays used by others (26). Our modifications permit direct detection of autoantibody-forming cells and allow us to determine isotype specificity of autoanti-IgG. This technique should be particularly useful for studying the RF response in vitro.

The total number of immunoglobulin idiotypes involved in the RF response studied here is potentially quite small because of the restricted nature of the antibody specificities produced. We know, however that the anti-IgG1 responses in a number of Ig4[a] allotype strains that we have studied is not idiotypically homogeneous because at least two subspecificities exist, one that recognizes Ig4[a] only and one that sees a determinant common to both Ig4[a] and Ig4[b]. No purely anti-Ig4[b] responses were observed in these mice. This is consistent with the idea that in hyperimmunized animals, immune complexes behave similarly to antigens of other types in the sense that they elicit an exquisitely specific antibody response. However the reason that autoantibody is produced against only some parts of the IgG1 molecule remains to be elucidated.

It is also curious that very little switching of IgM to IgG occurs in the anti-IgG1 response in hyperimmunized mice, even after multiple exposure to immune complexes during repeated immunizations. This type of immune response pattern is unusual, and is found primarily in experimental systems that utilize the so-called T cell-independent antigens (33, 34). It is possible that immune complexes can also behave like T-independent antigens.

Two alternative explanations for the anti-IgG1 specificity of the RF are possible:

(a) The expressed specificities may be determined by the relative numbers of B cells of different anti-IgG specificities present at the time of stimulation. In this case the anti-IgG1 specificity of the anti-IgG response of twice-immunized A/J mice would be due to the fact that, in these mice, virtually all of the B cells capable of reacting to the IgG portion of immune complexes are specific for IgG1. It has been demonstrated that in many cases B cell mitogens can induce the production of large amounts of RF (9–12). This is presumably because some RF specificities are highly represented in the B cell repertoire. One study showed quite clearly a nonrandom distribution of anti-IgG specificities found among a number of hybridoma lines derived from LPS-induced B cell blasts. Most of the anti-IgG clones were specific for IgG1 or IgG2a. However, it is possible that in this study the procedures used to produce and screen the hybridomas for anti-IgG activity could have contributed to this result (35).

(b) Alternatively, IgG1 may be inherently more autoimmunogenic than the other IgG isotypes and thus favor the production of autoanti-IgG1 despite the fact that the relative numbers of potentially reactive B cells specific for the various IgG subclasses may be comparable. This possibility appears to be plausible on the basis of indirect evidence. IgG1 differs fundamentally from other mouse IgG isotypes in that it does not fix complement (36). This fact is especially intriguing because of the demonstrable Fc specificity of the anti-IgG1 produced, and suggests that the immune complexes that induce RF production are those that have not fixed complement. It is unlikely that the IgG1 preference of the autoanti-IgG of antigen-boosted A/J mice is the result of the relative amounts of the various IgG subclass antibodies produced in response to the primary immunization with antigen, because relatively large amounts of IgG2a and IgG2b are produced along with IgG1 subsequent to immunizations with protein antigens in CFA (Fig. 3 and unpublished results).

Although it appears that the autoanti-IgG described here is induced by antigen:antibody complexes it is by no means clear that the resulting antibodies recognize conformational changes in the IgG due to antigen binding. Indeed, in two studies in which the relative affinities of the binding sites of human rheumatoid factors to native or aggregated IgG were checked, no significant differences were found (37, 38).

Although in a strict sense the autoanti-IgG1 response we have characterized is autoimmune since it is specific for self molecules, we believe that it differs from other autoantibody responses and is a result, rather than a cause, of disease. Similar views were put forward by Kunkel and Tan (39). Our results point to a simple explanation for the association of RF with autoimmune disease. Because production of anti-IgG1 is likely caused by immune complexes containing IgG1,

any situation in which both antigen and IgG1 antibody to the antigen are present will cause RF-like enhancers to be produced. In humans, RF-like antibodies can be found during severe infections but are usually present transiently, presumably because the antigen is cleared quickly (40, 41). This is analogous to the type of pattern we see in the experiment shown in Fig. 2. In autoimmune diseases in which antibodies are produced against self-determinants, new self antigens are constantly being produced or expressed. Thus immune complexes, either circulating or tissue bound, would be expected to be present transiently, presumably because the antigen is cleared quickly (40, 41). This is analogous to the type of pattern we see in the experiment shown in Fig. 2. In autoimmune diseases in which antibodies are produced against self determinants, new self antigens are constantly being produced or expressed. Thus immune complexes, either circulating or tissue bound, would be expected to be present chronically and RF-like enhancers would also be found consistently in response to these complexes. Since RF-like antibodies are generally IgM and fix complement efficiently, they could clearly contribute to the pathology without necessarily initiating it. This scheme fits relatively well with the known fact that the appearance of RF is closely correlated with both the duration and the intensity of a number of *different* autoimmune diseases (42, 43). It has been shown recently that passive transfer of anti-collagen type II antibody can induce rheumatoid arthritis in rats (44). We would predict the RF-like enhancer production rapidly follows the introduction of the anti-collagen antibody in these rats and that these two types of antibodies operate synergistically to destroy self tissue.

We have made the point elsewhere that autoanti-IgG may have both regulatory and effector roles in the normal immune response (4). These speculations seem all the more justified in view of the findings of this study pointing to the fact that, in terms of both quantity and frequency of appearance, autoreactive IgM anti-IgG1-producing cells in mice appear to be "normal." Clarkson and co-workers (45) have presented evidence indicating that RF-like materials may contribute to the ability of young rats to combat parasitic infections. This may be but one of many examples of the beneficial effects of RF-like enhancing antibody.

Summary

A/J mice were found to produce autoreactive IgM anti-IgG1 in response to secondary immunization with a number of protein antigens. No anti-IgG1 was produced after a single such immunization, indicating that antigen: IgG1 antibody complexes were responsible for inducing the autoreactive response. The size of the anti-IgG1 response was in some cases massive and of the same order of magnitude as the response to the foreign immunizing material. No significant anti-IgG2a, anti-IgG2b, or anti-IgG3 response was found in mice producing anti-IgG1. Virtually all of the anti-IgG1 material produced was of the IgM class and bound to the Fc region of autologous IgG1. A component of the anti-IgG1 was shown to be able to distinguish between the two mouse IgG1 allotypes.

These results suggest that self-reactive anti-IgG is a common component of the secondary immune response of mice that may have powerful physiological and immunoregulatory effects.

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