RHEUMATOID FACTOR (RF) PRODUCTION DURING ANAMNESTIC IMMUNE RESPONSES IN THE MOUSE

III. Activation of RF Precursor Cells Is Induced by Their Interaction With Immune Complexes and Carrier-specific Helper T Cells

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Several observations suggest that the Fc-specific anti-IgG autoantibodies, which were first discovered in the serum of rheumatoid patients (1, 2) and are therefore commonly called rheumatoid factors (RF),[1] may represent normal components of the immune system. RF are often detected, although usually in low titers, in the serum of normal individuals (3), and RF-like material has been found in healthy animals of various species, including hyperimmune rabbits and unmanipulated normal mouse strains (4–7). The existence of a "normal" RF synthesis thus seems well established, but the origin and the biological significance of this phenomenon remain largely unexplained.

The production of RF is considerably enhanced during active immunization against a wide variety of antigens. In humans, the frequency of RF precursor cells was found to increase after vaccination of normal adults with tetanus toxoid (8) and, in the mouse, it was observed that anamnestic immune responses to protein antigens invariably started with the production of considerable amounts of RF (9, 10). In addition, in the mouse model, it was shown that exposure to antigen initiated RF synthesis in primed animals only, which implied that the activation of RF precursor cells required some kind of interaction between antigen and primed lymphocytes and/or immune serum components. Support for this hypothesis was obtained in adoptive transfer experiments, which demonstrated that both primed lymphocytes and immune serum were involved in the process of RF production (11). We have examined these humoral and cellular mechanisms of RF induction in greater detail.

Materials and Methods

Mice. C57BL/6 and BALB/c mice were derived from breeders originally provided by Dr J. L. Guénet (Institut Pasteur, Paris). For some experiments, additional animals were purchased from Iffa Credo (Les Oncins, France). The latter mice were maintained under a sterile airflow and given sterile food and water.

This work was supported by the Fonds de la Recherche Scientifique Médicale, Fonds National de la Recherche Scientifique (FNRS), and Loterie Nationale, Belgium. P. Coulie is a research assistant and J. Van Snick a research associate with FNRS.

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; RF, rheumatoid factor; RIA, radioimmunoassay; TNP, trinitrophenyl.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/85/1/0088/10 $1.00
Volume 161 January 1985 88–97

Antigens and Immunizations. Human transferrin (Behringwerke AG, Marburg/Lahn, Federal Republic of Germany) and keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., La Jolla, CA) were trinitrophenylated (TNP) by treatment with 2,4,6,-trinitrobenzenesulfonic acid (Eastman Kodak Co., Rochester, NY). For TNP-KLH, the subscripts refer to the number of TNP residues per 100,000 daltons. For TNP-transferrin, they correspond to the number of residues per molecule.

For immunization, 50 μg protein was emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) and injected subcutaneously into 7–10-wk-old female C57BL/6 mice. After 14 d, the animals received a second injection, and 2–6 wk later they were used as spleen cell donors.

Reconstitution Experiments, Culture Conditions, and Cell Preparations. 6–8-wk-old female C57BL/6 mice were irradiated with 900 rad from a Cs source 8–24 h before intravenous transfer of $3–4 \times 10^7$ spleen cells. 18–24 h later they were challenged intravenously with antigen or immune complexes in 0.5 ml saline. After 5 d, the spleen cells of these animals were cultured for 24 h as described (11), and the supernatants were tested for RF activity.

Spleen cells treated with an AKR anti-Thy-1.2 monoclonal antibody (mAb) (A5703A8; γ2b, κ) and guinea pig complement were used as a source of B cells. Enriched T cell populations were obtained by nylon wool filtration (12). Serological analysis of these populations was performed by standard indirect immunofluorescence procedures.

RF and Antibody Determination. IgM anti-IgG1 and IgM anti-IgG2a responses were measured by radioimmunoassay (RIA) using polyvinyl wells coated with IgG1 or IgG2a mAb as described (7). Since the RF produced by C57BL/6 mice have no detectable allotypic specificity, proteins of C57BL/6 or 129/Sv origin were used indifferently. Anti-KLH antibodies were measured by a similar RIA. The assays were calibrated with purified mAb.

Antibodies. Hyperimmune ascites against KLH and human transferrin were induced in C57BL/6 mice by weekly intraperitoneal injections of 100 μg antigen in 1 vol isotonic saline emulsified in 9 vol CFA. These ascites contained substantial amounts of RF which were removed by passage over mouse IgG–Sepharose. For the anti-KLH ascites, any IgM RF that may have escaped absorption on the IgG column was further removed by gel filtration on an Ultrogel AcA-22 column (LKB Produkter, Bromma, Sweden). Specific antibodies were then isolated by affinity chromatography on Sepharose columns to which antigens had been coupled by the cyanogen bromide method. Elution was performed with a 0.05 M sodium citrate buffer at pH 2.6. The purified antibodies, which were >90% IgG1, were ultracentrifuged on isokinetic sucrose gradients, and only 7 S fractions were used (13).

mAb were obtained by fusion of spleen cells from appropriately immunized mice with SP2/0-Ag14 cells (14) and isolated by chromatography on protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Unless otherwise stated, all immune complexes were made at equivalence or at a molar antigen/antibody ratio of 1:1, and incubated for 2 h at 37°C before use.

Results

Immune Complexes Induce RF in Antigen-primed Mice. We tested the ability of immune complexes (formed in vitro with two different antigens, KLH and human transferrin) to induce the production of RF in irradiated C57BL/6 mice adoptively transferred with syngeneic spleen cells primed to the appropriate antigen. The antigens were combined at equivalence with affinity-purified antibodies that were isolated from hyperimmune syngeneic ascites and cleared of aggregated material by ultracentrifugation. In both systems, injection of either antigen or 7 S antibody alone elicited little or no RF production. By contrast, <10 μg complexed antibody induced a considerable RF response (Table I). Similar results were obtained with IgG1 anti-KLH mAb of 129/Sv origin (B8605C5). In this

TABLE I

RF Induction by Preformed Immune Complexes

Transferred spleen cells*	Antigen	Antibody	IgM anti-IgG1
	μg	*μg*	*ng/ml*
Transferrin-primed	60	—	27 (1.45)‡
	—	200	19 (1.38)
	60	200	191 (1.20)
	6	20	170 (1.10)
	0.6	2	82 (1.41)
KLH-primed	100	—	2.9 (1.36)
	—	300	2.4 (1.21)
	100	300	238 (1.14)
	10	30	106 (1.34)
	1	3	163 (1.12)

* Irradiated (900 rad) C57BL/6 mice were adoptively transferred with transferrin- or KLH-primed syngeneic spleen cells as described in Materials and Methods. After 24 h, the reconstituted animals received an intravenous injection of antigen and/or affinity-purified antibody. When administered together, antigen and antibody were combined at equivalence for 2 h at 37°C before injection. 5 d later spleen cells were cultured for 24 h at 10^7 cells/ml, and the concentration of IgM anti-IgG1 was measured by RIA of the culture supernatant.

‡ Log mean and standard error (five mice per group).

system, a significant RF response was detected with only 1 μg of complexed antibody (data not shown).

RF Induction as a Function of Antigen/Antibody Ratio in Immune Complexes. Complexes with different antigen/antibody ratios were made by incubating varying amounts of KLH with a constant amount of antibody. To test the influence of extreme antigen excess without injecting milligram amounts of KLH, we used two doses of antibody. For each group, complexes with antigen/antibody ratios close to equivalence were included for the sake of comparison. As shown in Table II, RF responses were drastically reduced after challenge with complexes in large antigen excess. A similar observation was made with polyclonal antitransferrin antibodies (data not shown).

Specificity of RF Induction by Immune Complexes. We tested the RF-inducing capacity of complexes formed with $F(ab')_2$ fragments derived from the IgG1 anti-KLH mAb used in the preceding experiments. Although these fragments precipitated KLH, they did not induce any detectable production of IgG1-specific RF (Fig. 1). Precipitating complexes formed with IgM mAb likewise failed to induce any anti-IgG1 RF production (Fig. 1), emphasizing the requirement for IgG1-specific Fc epitopes in the complexes.

Enhanced RF Production After Immunization With Preformed Immune Complexes. IgG2a-specific RF have not been detected in anamnestic responses to protein antigens (9–11), and, in the adoptive transfer system used here, IgG2a complexes induced only weak anti-IgG2a responses (data not shown). Because this could be due to the low frequency of IgG2a-specific precursors in the spleen cell populations used to reconstitute the animals, we immunized BALB/c mice by weekly intraperitoneal injections of TNP-KLH, either alone or complexed

TABLE II

RF Induction by Preformed Monoclonal Immune Complexes: Influence of Antigen/Antibody Ratio

Exp.	KLH	IgG1 anti-KLH	Ag/Ab* ratio	IgM anti-IgG1
	μg	*μg*		*ng/ml*
1	1.5	50	0.03	212 (1.62)‡
	5	50	0.1	276 (1.41)
	50	50	1	331 (1.51)
	10	10	1	151 (1.12)
	100	10	10	144 (1.08)
	300	10	30	38 (1.33)
	1.5	—	—	16 (1.48)
	300	—	—	11 (1.17)
2	3	10	0.3	191 (1.35)
	30	10	3	291 (1.17)
	300	10	30	27 (1.29)
	30	—	—	10 (1.30)
3	10	10	1	209 (1.45)
	300	10	30	10 (1.05)

Irradiated (900 rad) C57BL/6 mice were adoptively transferred with KLH-primed syngeneic spleen cells. After 24 h, the animals were challenged intravenously with KLH or preformed (2 h at 37°C) immune complexes containing KLH and IgG1 anti-KLH mAb (B8605C5). 5 d later, spleen cells were cultured for 24 h, and IgM anti-IgG1 levels were measured by RIA of culture supernatants (as described in Materials and Methods).

* Ag/Ab (antigen/antibody) ratios are given on a wt/wt basis. Complexes made at equivalence had a ratio of 1:1.

‡ Log mean and standard error (three to four mice per group).

with IgG1 or IgG2a anti-TNP mAb of BALB/c allotype. As shown in Fig. 2, immunization with complexes resulted in much higher RF levels than immunization with antigen alone. In addition, in these experiments, both IgG1 and IgG2a complexes elicited a strong isotype-specific RF response.

Involvement of T Cells in RF Production. The possible involvement of T cells in the activation of RF precursor cells was assessed by testing the ability of T cell–deprived, primed spleen cells to produce RF after challenge with immune complexes. As shown in Table III, removal of T cells not only prevented the development of a secondary response to KLH, but also abolished any RF production.

These data strongly supported the idea that T cell activation was required for immune complexes to stimulate RF precursor cells. It was not clear, however, whether B cell priming was also necessary in this process. We therefore measured the RF response of irradiated C57BL/6 mice reconstituted with various combinations of KLH-primed and unprimed B and T cells. As shown in Table IV, optimal RF production was observed in mice adoptively transferred with primed T cells and naive B cells, which demonstrated that normal B cell populations

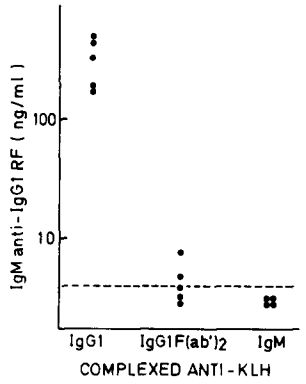

FIGURE 1. Immunoglobulin determinants involved in RF induction. Irradiated C57BL/6 mice were reconstituted intravenously with KLH-primed syngeneic spleen cells and challenged intravenously 1 d later with 30 μg KLH complexed at equivalence with IgG1 (B8605C5), IgG1 F(ab')$_2$ fragments, or IgM (B9402A3) anti-KLH mAb. The RF response of individual animals was measured 5 d later in 24-h spleen cell cultures. The dotted line represents the detection limit.

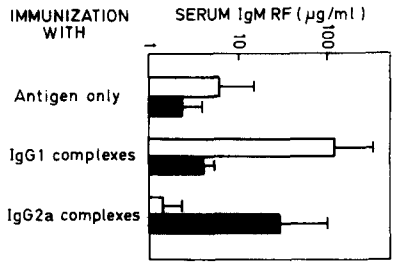

FIGURE 2. Induction of isotype-specific RF by immunization with immune complexes. IgM anti-IgG1 (□) and IgM anti-IgG2a (■) concentrations in the serum of BALB/c mice after four weekly intraperitoneal injections of 10 μg TNP_4-KLH, either in a free form or complexed with IgG1 (B8401H5) or IgG2a (A6202F4) anti-TNP mAb (five mice per group; log mean and two standard errors).

TABLE III

Absence of RF Production in T Cell–deprived Primed Spleen Cells Stimulated with Immune Complexes

Spleen cell treatment	Number of mice	IgM anti-IgG1 RF	IgM anti-KLH	IgG1 anti-KLH
Complement alone	6	901 (1.10)	1,098 (1.06)	44,180 (1.06)
Anti-Thy-1.2 plus complement	9	10 (1.28)	31 (1.20)	520 (1.17)

Irradiated (800 rad) C57BL/6 mice were reconstituted intravenously with the equivalent of 3.5 × 10^7 syngeneic KLH-primed spleen cells treated with guinea pig complement alone, or with anti-Thy 1.2 mAb plus complement. 1 d later, the mice were injected intravenously with 30 μg KLH complexed with an equivalent amount of IgG1 anti-KLH mAb (B8605C5). The complexes were incubated for 2 h at 37°C before injection. Antibody responses were measured 5 d later by RIA of 24-h spleen cell cultures. The log means of antibody concentrations are given in ng/ml (standard error in parentheses).

TABLE IV

Influence of B or T Cell Priming on RF Induction in Adoptively Transferred C57BL/6 Mice

B cells*	T cells*	Number of mice	IgM anti-IgG1
			ng/ml
Naïve	Naïve	6	37 (1.41)‡
Naïve	Immune	7	239 (1.32)
Immune	Naïve	8	63 (1.23)
Immune	Immune	7	203 (1.29)

6–8-wk-old normal female C57BL/6 mice were irradiated with 900 rad and adoptively transferred 2 h later with various combinations of syngeneic B (2×10^7) and T (10^7) cells from naïve or KLH-primed mice. 1 d later, they were challenged intravenously with preformed (2 h at 37°C) immune complexes prepared at equivalence with 50 μg KLH and 50 μg IgG1 anti-KLH mAb (B8605C5). After 5 d, spleen cells were cultured for 24 h and IgM anti-IgG1 levels measured by RIA of culture media.

* Preparation and serologic analysis of B and T cell populations were as described in Materials and Methods. Percentages of Ig- and Thy-1.2-positive cells were as follows: 63 and 4 for naïve B cells, 61 and 3 for immune B cells, 4 and 74 for naïve T cells, and 3 and 70 for immune T cells, respectively.

‡ Log means (standard error in parentheses).

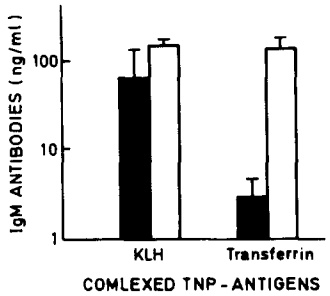

FIGURE 3. Carrier-specificity of spleen cell priming in RF induction. Irradiated C57BL/6 mice were reconstituted intravenously with TNP-KLH–primed syngeneic spleen cells and challenged intravenously 1 d later with 10 μg KLH plus 75 μg IgG1 anti-TNP mAb (B8401H5) complexed with 20 μg of either TNP_5-KLH or TNP_4-transferrin. IgM anti-IgG1 (■) or IgM anti-KLH (□) responses of individual recipients were measured 5 d later in 24-h spleen cell cultures. Bars represent the log means plus two standard errors.

contained sufficient numbers of RF precursor cells to mount optimal RF responses. This result was not due to contamination of our primed T cells with small numbers of RF-secreting B cells, because RF production by mice reconstituted with primed T cells alone did not exceed background levels (data not shown).

Specificity of the T Cell–mediated Activation of RF Precursor Cells. We compared the RF responses of primed spleen cells, stimulated in vivo with immune complexes containing the priming antigen or an unrelated antigen. Irradiated C57BL/6 mice, adoptively transferred with TNP-KLH-primed spleen cells, were challenged with IgG1 anti-TNP mAb complexed to either TNP-KLH or TNP–human transferrin. The complexes were injected along with free KLH to ensure a normal secondary response in both groups. As shown in Fig. 3, substitution of

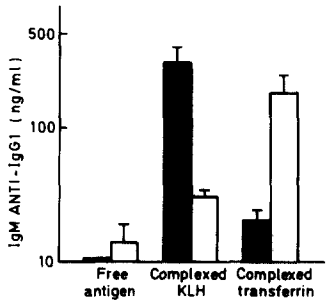

FIGURE 4. Antigen-specificity of spleen cell priming in RF induction. Irradiated C57BL/6 mice were reconstituted intravenously with KLH (■)- or transferrin (□)-primed syngeneic spleen cells. 1 d later, they were challenged intravenously with the priming antigen in a free form (50 μg transferrin or 20 μg KLH), or with the same amount of KLH or transferrin complexed with IgG1 anti-KLH mAb (B8605C5) or polyclonal affinity-purified antitransferrin antibodies. When the heterologous antigen was combined with antibody, the homologous antigen was also injected, in a free form, to generate a normal secondary response. The RF response of individual recipients was measured 5 d later in 24-h spleen cell cultures. Bars correspond to log means plus two standard errors of IgM anti-IgG1 levels in culture supernatants.

transferrin for KLH in the complexes almost completely abolished RF production. Similar results were obtained in criss-cross experiments using mice reconstituted with spleen cells primed to KLH or human transferrin and challenged with complexes containing either antigen (Fig. 4).

Discussion

During the early stages of murine secondary immune responses to protein antigens, a large proportion of the antibody-secreting cells produce IgM specific for constant region determinants of mouse IgG1 (9–11). The importance of the immune status of the animal in the development of this RF production is underscored by the absence of RF synthesis during primary responses to the same antigens. Surprisingly, antigen-primed spleen cells transferred into naive irradiated animals do not support RF production upon challenge with antigen unless immune serum is transferred along with the cells (11), suggesting that immune complexes may be involved in the activation of murine RF precursor cells.

Formal proof for this hypothesis was obtained by measuring the RF responses of irradiated mice reconstituted with antigen-primed spleen cells and challenged with preformed immune complexes. Whereas antigen or antibody injected alone consistently failed to elicit significant RF production in these animals, they proved to be potent RF inducers when injected together, with as little as 1 μg of complexed antibody being sufficient to generate significant RF synthesis.

The RF that are produced during anamnestic responses to protein antigens have a remarkable specificity for the IgG1 subclass (9, 10). It was not known whether this specificity resulted from a selective stimulation of anti-IgG1 precursor cells or whether it simply reflected the higher frequency of these cells in the mouse B cell repertoire. The use of mAb of different isotypes, or of fragments

derived from them, enabled us to show that the IgG1 specificity of RF depends strictly upon the presence of IgG1 Fc determinants in the complexes. Indeed, when IgG1 $F(ab')_2$ fragments, IgM, or IgG2a were substituted for IgG1 in the complexes, the IgG1-specific RF production was drastically reduced. Activation of RF-secreting cells thus apparently requires a specific interaction between the RF-producing cell and Fc region determinants in the immune complex.

The magnitude of the RF response generated by such interactions obviously depends upon the frequency of RF cells of appropriate specificity. It is likely that the much weaker anti-IgG2a response generated by IgG2a complexes reflects the lower frequency of IgG2a-specific RF cells in the spleen cell populations used here. This limitation was, however, overcome by repeatedly immunizing animals with preformed IgG2a complexes; a strong and specific anti-IgG2a RF response developed after such treatment. Like other immune cells, lymphocytes secreting RF seem thus to be specifically recruited by repeated stimulation with their antigen.

As mentioned above, IgG1 immune complexes fail to induce significant anti-IgG1 RF responses in unprimed mice, indicating that priming of the immune system with a foreign antigen somehow governs the capacity of immune complexes to activate RF precursor cells (11). The results reported here demonstrate that the enhanced RF response of primed lymphocytes is chiefly due to the priming of helper T cells. Removal of T cells from primed spleen cell populations completely abolished anti-IgG1 RF production, whereas the reconstitution of irradiated mice with primed T cells and naive B cells resulted in maximal RF synthesis. The latter finding emphasizes the high frequency of IgG1-specific RF precursor cells in adult spleen cell populations, which had already been noted after polyclonal activation with lipopolysaccharide (15).

Stimulation of primed T cells with free antigen did not promote the activation of RF precursor cells by immune complexes of unrelated antigen. The most likely explanation for this observation is that carrier-specific helper T cells activate RF B cells via an antigen-antibody bridge where antibody plays the same role as the haptenic group in hapten-carrier–mediated T-B interactions. Although we favor the idea that specific helper T cells act directly on the RF-secreting B cell, our data do not formally exclude the possibility that this phenomenon involves yet another T cell with specificity for Ig determinants.

The involvement of xenoantigen-specific T cells in the control of RF responses is not an isolated example of the ability of these cells to contribute to the development of immune reactions against determinants of self-IgG. Carrier-specific helper T cells have been found which cooperate in the production of auto-antiidiotypic antibodies in mice immunized with complexed antibody (16). These observations raise the possibility that helper T cells reactive with non-self antigens may also control the development of autoimmune reactions to molecules other than antibodies. In this vein, it is particularly intriguing that certain autoimmune reactions induced in the mouse by infections with reoviruses only affect those tissues where the viruses actually multiply (17).

Summary

IgG1 immune complexes were identified as the humoral stimuli responsible for the synthesis of IgG1-specific IgM rheumatoid factor (RF), which occurs in the mouse during the early stages of secondary immune responses to protein antigens. The specificity of this phenomenon was illustrated by the fact that complexes made with IgG1 $F(ab')_2$ fragments or with antibodies of a different isotype failed to induce significant anti-IgG1 RF synthesis. The importance of immune complexes in the induction of RF was further underscored by the substantial increase in the titers of isotype-specific RF observed in the serum of mice immunized with IgG1- or IgG2a-complexed antigen rather than with antigen alone. The RF-inducing capacity of the complexes varied with the antigen/antibody ratio: it was maximal in antibody excess or at equivalence, but dramatically reduced in large antigen excess.

The importance of T cell priming in RF precusor cell activation by immune complexes was demonstrated by the failure of T cell–deprived spleen cells to reconstitute the capability of irradiated mice to produce RF, and by the optimal RF responses observed after reconstitution of irradiated recipients with primed T cells and naive B cells. The involvement of T cells in this process could not be explained by the release of nonspecific B cell activators, because antigenic stimulation of primed T cells failed to enhance the activation of RF precursor cells by immune complexes of unrelated antigen.

We thank Mrs. M. Stevens and E. Hanssens for expert technical assistance, and Mr. M. Delory and Mrs. N. Joris for skillful typing of this paper. We gratefully acknowledge the support of Professor P. Masson.

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