Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 613–615, January 1982
Immunology

Biological activities of antitrinitrophenyl and antidinitrophenyl mouse monoclonal antibodies

(passive cutaneous anaphylaxis/complement fixation/properties of mouse immunoglobulins)

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Communicated by Lloyd J. Old, October 7, 1981

ABSTRACT **The biological activities of mouse IgG1, IgG2a, IgG2b, and IgE monoclonal antibodies were investigated. As has been shown with conventional antibodies, monoclonal IgG1 and IgE are the two classes capable of sensitizing the mouse for anaphylactic reactions, IgE sensitizes the rat, and IgG2a sensitizes the guinea pig. What has not been seen with conventional antibodies is that large amounts of monoclonal IgG2a or IgG2b can sensitize the mouse, very large amounts of monoclonal IgE can sensitize the guinea pig, and very large amounts of IgG1 and IgG2a can sensitize the rat. One of the IgG1 monoclonal antibodies was capable of fixing complement by the classical pathway.**

The use of monoclonal antibodies facilitates investigation of the biological activities of immunoglobulin molecules with well-defined specificities; in many cases, separation of the different mouse immunoglobulin isotypes is exceedingly difficult. We investigated the sensitizing capacity for homologous and heterologous animal species and the complement-fixing ability by the classical pathway of mouse immunoglobulins with trinitrophenyl (TNP) or dinitrophenyl (DNP) specificities of the IgG1, IgG2a, IgG2b, and IgE classes produced by hybridomas.

MATERIALS AND METHODS

The creation of hybridomas for production of monoclonal antibodies has been described (1). The antibody-producing parent cells were taken from spleens of BALB/c mice immunized with TNP-protein (1, 2) or from spleens of BALP/c mice immunized with DNP-protein [IgE anti-DNP hybridoma (3)]. One of the original anti-DNP hybridoma lines was recloned several times; that the progeny secrete a monoclonal anti-DNP IgE antibody (clone B.53) is shown by the fact that it was neutralized by a specific anti-idiotypic antibody (4).

Hybridomas designated DO- were produced by fusion of cell line P3X63Ag8653 (a gift of S. Sawada, Nihon University, Tokyo) with spleen cells from BALB/c mice. These mice were immunized by intraperitoneal injection of 100 μg of DNP-keyhole limpet hemocyanin with 8 mg of alum and 10^9 *Bacillus pertussis* organisms and boosted 2 weeks later with 100 μg of DNP-keyhole limpet hemocyanin. Spleen cells were harvested 3 days after boosting. (BALB/c × A/J) (CAF_1) mice were used for production of anti-DNP hybridomas designated NK as described (5).

All the hybridomas were recloned several times and tested for production of monoclonal antibodies. For *in vivo* propagation, 10^6 hybridoma cells were injected intraperitoneally into 6-week-old female BALB/c or CAF_1 mice. Ascites were harvested 6 to 8 days later, centrifuged, and stored at −20°C.

For purification, ascites were passed through a column made by coupling 27 mg of DNP_{34}-bovine serum albumin to 2 g of Sepharose 4B (6), and the antibody was eluted with a double volume of DNP-ε-aminocaproic acid (1.5 mg/ml) in phosphate-buffered saline, pH 7 or 8, according to the isoelectric point of the antibody. The eluted antibody was thoroughly dialyzed with the same phosphate-buffered saline and concentrated to one-fifth of the initial volume. Less than 1% remained on the DNP_{34}-bovine serum albumin-Sepharose 4B columns. In some cases, the column was washed with 2M glycine·HCl, pH 2.4, and 2.75-ml aliquots were taken up in 0.8 ml of 0.5 M Tris buffer, pH 8.6, and then dialyzed against phosphate-buffered saline. Micro-Kjeldahl analysis was carried out (7) to determine the protein content of the purified antibodies.

Antigens. DNP_{34}-bovine serum albumin and DNP_9-egg (hen) albumin were the same preparations used in previous experiments (8).

TNP_{15}-egg albumin and TNP_{36}-bovine serum albumin were prepared similarly to the DNP derivatized proteins except that TNP-sulfonic acid (Eastman) was used instead of DNP-sulfonic acid and the coupling was done in 0.2 M borate buffer, pH 9.

Animals. English short hair guinea pigs of both sexes, 250–300 g; Sprague–Dawley male rats, 350 g (Camm Research Institute, Wayne, NJ); and CFW mice (Charles River Breeding Laboratories) were used for passive cutaneous anaphylactic (PCA) reactions. BALB/c (Cumberland Farms, Clinton, TN) and CAF_1 mice (Jackson Laboratory) were used to maintain the hybridomas *in vivo*. All mice were female and 6 weeks old.

Biological Assays. Passive hemolysis was carried out as described (9) with TNP_{36}-bovine serum albumin or TNP- (10) or DNP- (11) coated sheep erythrocytes. PCA as described (12, 13) was used to investigate the sensitizing capacity of the different monoclonal antibodies in mice, guinea pigs, and rats. The sensitizing period was 90 min for mice except for IgE when it was 2 days, 3 to 4 hr for guinea pigs, and 2 hr for rats. Mice and guinea pigs were challenged with 500 μg of DNP_9-egg albumin or TNP_{15}-egg albumin and rats were challenged with 1 mg of DNP_{34}-bovine serum albumin. Affinity measurements were done as described (14).

Rabbit Anti-Mouse Immunoglobulins. All hybridomas were tested with anti-IgG1, -IgG2a, and -IgG2b, anti-λ, and anti-κ antibodies from Litton Bionetics (Kensington, MA). In addition, they were tested with much more potent rabbit anti-immunoglobulins prepared in our laboratory against purified MOPC 21 (IgG1), UPC 10 (IgG2a), MOPC 195 (IgG2b), and B.53 (IgE) and rendered specific by crossabsorption on Sepharose 4B columns to which the appropriate immunoglobulin

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Abbreviations: DNP, dinitrophenyl; PCA, passive cutaneous anaphylaxis; TNP, trinitrophenyl.

(15 mg/g of Sepharose 4B) was coupled. These precipitated in double-diffusion (Ouchterlony) tests even when diluted 1:80 or 1:160 and precipitated only the appropriate isotype.

Anti-IgG1 and anti-IgG2a were also used to prepare immunoabsorbent columns. The antisera were precipitated at 40% saturation with $(NH_4)_2SO_4$ and dialyzed, and 15 mg of protein was coupled to 1 g of Sepharose 4B (6).

RESULTS

All IgG1 monoclonal antibodies gave one line in double diffusion tests in agar (Ouchterlony plates) against DNP-bovine serum albumin, therefore the possibility of a mutant antibody with only one combining site (2) can be excluded because precipitation requires a bivalent antibody. The anti-DNP IgG2b (NK) does not precipitate and does not give a line against DNP-bovine serum albumin in double diffusion, but it is bivalent because the valency extrapolates to 2 (N. Klinman, personal communication). All ascites were examined by double diffusion in agar against rabbit antiheavy chain antibodies (antisera from Bionetics and also our own preparations). Thus, DO5-1G1 and NK IgG1 reacted only against anti-IgG1, DO5-1C4 and DO5-1A7 reacted against anti-IgG2a, NK IgG2b reacted against anti-IgG2b and B.53 reacted against anti-IgE. Contamination of the IgG1, IgG2a, or IgG2b monoclonal antibodies by small amounts of IgE was excluded by reverse PCA reactions (12) in rats with specific rabbit anti-IgE. Whereas 10 ng of IgE gave strong reverse PCA reactions, no reaction was obtained with any of the monoclonal antibodies of the other classes, even when milligram amounts were injected intradermally (not shown). Sera were diluted with phosphate-buffered saline or Veronal or both buffer for passive hemolysis and doubling serial dilutions were used. Preliminary experiments were done starting with 1:2 dilutions. Subsequently, we started with much higher dilutions. Initially, in 1978, hemolysis was carried out with anti-TNP monoclonal antibodies and TNP_{36}-bovine serum albumin-coated sheep erythrocytes. Later we realized that TNP_{36}-bovine serum albumin-coated sheep erythrocytes are much less effective targets for monoclonal anti-TNP antibodies than those coated directly with TNP, as described in ref. 10. However, because these monoclonal antibodies had been prepared in the Basel Institute of Immunology and our supply of Hy 1.2 (IgG2a) was exhausted when we realized that direct TNP coating gives a more effective target, we could not repeat these experiments with sheep erythrocytes coated directly with TNP. This explains the lower hemolytic titers of anti-TNP monoclonal antibody Hy 1.2 (IgG2a) (Table 1).

Results of representative experiments are given in Table 1. Every titration was repeated at least three times. The hemolytic titers for anti-TNP antibodies were obtained with TNP_{36}-bovine serum albumin-coated sheep erythrocytes for Hy 1.2 and TNP-coated sheep erythrocytes for GK-14-8-8-12.

There was some slight variation among experiments, but never more than one dilution from that shown in Table 1. No antibody gave a specific reaction at some times but not at others.

Only the endpoint of the passive hemolysis (the last dilution giving 100% lysis) and the last dilution giving a PCA reaction are shown in Table 1.

Monoclonal anti-TNP IgG2a (Hy 1.2)-sensitized mice for PCA reactions, and this was an unexpected result. For this reason, aliquots were absorbed on anti-IgG1 and also on anti-IgG2a immunoabsorbent columns, concentrated to the initial volume, and tested again. The anti-IgG2a column absorbed all activities, whereas the anti-IgG1 column did not absorb any of them (Table 1). It was concluded, therefore, that this monoclonal IgG2a (Hy 1.2) can sensitize mice for PCA reactions.

Table 1: Reactions with monoclonal antibodies

Monoclonal antibody	PCA			Passive lysis	Affinity (K_a), M^{-1}
	Mouse	Guinea pig	Rat		
		Anti-TNP			
Hy 2.15 IgG1 (κ)	800	ND	ND	ND	ND
Hy 1.2 IgG2a (κ)	1,600	12,800	ND	800	ND
Hy 1.2 IgG2a (κ) absorbed on anti-IgG1 column	1,600	12,800	ND	800	ND
Hy 1.2 IgG2a (κ) absorbed on anti-IgG2a column	0	0	ND	0	ND
GK-14-1-8-8-13 IgG2b (κ)	400	ND	ND	6,400	ND
		Anti-DNP			
DO5-1G1 IgG1 (κ)	8,000	0	500	40,000	3×10^7
DO3-2G6 IgG1	4,000	ND	100	0	1×10^8
DO5-1C4 IgG2a (κ)	16,000	160,000	10	160,000	3×10^8
DO5-1A7 IgG2a (κ)	2,000	40,000	0	80,000	3×10^8
NK IgG1 (κ)	4,000	0	0	0	ND
NK IgG2b (κ)	100	0	0	5,000	ND
B.53 IgE (κ)	80,000	100	80,000	0	3×10^8

PCA and passive lysis results represent the inverse of the last dilution giving a reaction. Passive lysis was done with sheep erythrocytes coated with TNP_{36} bovine serum albumin for Hy 1.2 monoclonal antibodies, with sheep erythrocytes coated with TNP for monoclonal antibody GK-14-1-8-8-13, and for all others with sheep erythrocytes coated with DNP.

ND, not done.

DO5-1G1, the complement-fixing IgG1, and NK IgG1, a noncomplement-fixing IgG1, DO5-1C4 and DO5-1A7, both IgG2a and B.53 (IgE) monoclonal antibodies were purified and the minimal amount of protein giving a threshold reaction is given in Table 2. Threshold PCA reactions with the antibodies eluted by the glycine·HCl (pH 2.4) buffer were obtained with the same amount of protein as the antibodies eluted by DNP-ε-aminocaproic acid (not shown).

DISCUSSION

Our results show that the IgG1 monoclonal antibody is the most effective IgG immunoglobulin isotype to sensitize the homol-

Table 2. Threshold reactions with purified monoclonal antibodies

Monoclonal antibody	PCA		
	Mouse, μg/ml	Guinea pig, μg/ml	Rat, ng/ml
DO5-1G1 IgG1	3 to 4	ND	ND
NK IgG1 IgG1	6 to 7	ND	ND
DO5-1C4 IgG2a	2.5–3	0.25–0.3	ND
DO5-1A7 IgG2a	4 to 5	0.25–0.4	ND
B.53 IgE	0.002–0.004	ND	2–4

ND, not done.

ogous species for PCA and that the IgG2a monoclonal antibody is the most effective to sensitize the guinea pig, as it was seen with conventional antibodies (15, 16). IgE, as expected, does not fix complement by the classical pathway and is the most potent immunoglobulin class to sensitize the mouse and the rat. Both IgG2a and IgG2b monoclonal antibodies fix complement well by the classical pathway.

A striking observation is that one of the IgG1 monoclonal antibodies (DO5-1G1) does fix complement well by the classical pathway, as shown by the passive lysis experiments, whereas some others (DO3-2G6 and NK IgG1) do not produce lysis at all. All three IgG1 monoclonal antibodies precipitate and give only one line against DNP_{34}-bovine serum albumin on Ouchterlony plates. The affinities of DO5-1G1 and DO3-2G6 are of the same order (Table 1). The minimal amount for threshold PCA reactions of DO5-1G1 and NK IgG1 are of the same order (Table 2).

It was thought several years ago that, just as guinea pig IgG1 (9, 17) does not fix complement, so also mouse IgG1 is not a complement-fixing antibody (15). Recently, however, it has been shown that this is not always the case; in the mouse, two varieties of IgG1 may exist, one that does not fix complement by the classical pathway and one that does (18, 19). The existence of monoclonal antibodies of the IgG1 class with complement-fixing capacities, in addition to other IgG1 monoclonal antibodies that do not fix complement, confirm the observations made with conventional antibodies. It must be emphasized that no differences were found between complement-fixing and noncomplement-fixing IgG1 monoclonal antibodies with respect to their precipitating activity and sensitizing capacities. Moreover, their affinities were similar.

It has long been known that mouse IgG2a can sensitize the guinea pig for anaphylactic reactions (16). It is now apparent that the IgG2a antibody is also capable of sensitizing the mouse for PCA reactions, although it is much less effective than for guinea pig. IgG2b is also capable of sensitizing the mouse for PCA, although we have found that its sensitizing capacity is much less when compared with its complement-fixing capacity: 1/400 vs 1/6400 in one case and 1/100 vs 1/5000 in the other (Table 1).

It was thought that the only mouse immunoglobulin isotype capable of sensitizing the rat is IgE (20). With the very high titer monoclonal antibodies, this seems to be not quite exact. High concentrations of IgG1 may give slight PCA reactions in rats. Very high concentrations of mouse IgE monoclonal antibody could also sensitize the guinea pig for PCA reactions, and very high concentrations of IgG2a can give slight PCA reactions in the rat. For practical purposes, however, these very slight PCA reactions (with IgG1, IgG2a, and IgE) have no great importance with conventional antisera.

We thank Dr. Norman Klinman of the Scripps Clinic and Research Foundation for lines of NK IgG1 and NK IgG2b, which we obtained from Dr. Victor Nussenzweig, New York University, Department of Pathology; Dr. Takeshi Tokuhisa, Chiba University, Chiba, Japan for performing the affinity measurements; and Mrs. Susanna Fekete de Nagyivany and Mr. Csaba de Szalay for their skillful technical assistance. This work was supported by National Institutes of Health Grants AI-03075 and CA-16247.

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