MICROBIOLOGY AND IMMUNOLOGY

Rapid Clearance of Rhesus-Positive Erythrocytes with Monoclonal Anti-Rhesus Antibodies Is Insufficient for Effective Prevention of Rhesus Sensitization

N. I. Olovnikova, E. V. Belkina, N. I. Drize, L. N. Lemeneva, G. Yu. Miterev, T. L. Nikolaeva, and I. L. Chertkov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 129, No. 1, pp. 77-81, January, 2000
Original article submitted October 16, 1998

The ability of human monoclonal IgG1 to accelerate the clearance of rhesus-positive erythrocytes in rhesus-negative volunteers does not correlate with their immunosuppressive effect. Monoclonal antibodies G-17 weakly accelerated the clearance, but decreased the incidence of sensitization, while G-7 and G-12 antobodies eliminated 80-100% erythrocytes for 7 days, and used in combination, these antibodies eliminated all erythrocytes within 3 days. G-7 antibodies stimulated immune response in all doses, while G-12 antibodies stimulated anti-rhesus response in a dose of 600 μg and notably decreased it in a dose of 1200-1800 μg.

Key Words: *rhesus sensitization; monoclonal anti-rhesus antibodies; erythrocyte clearance; flow cytometry*

Injections of anti-rhesus immunoglobulin to a rhesus-negative (Rh^-) woman within 3 days after delivery of a rhesus-positive (Rh^+) child 5-10-fold decreases the probability of rhesus sensitization and newborn hemolytic disease in subsequent pregnancies [3,8]. The mechanism of prevention of immunization with passive anti-Rh antibodies is unclear. It is believed that the preventive effect depends on the rate of elimination of Rh^+ erythrocytes (ER) from the circulation and their destruction in the spleen. Experimental findings indicate that immune response can be effectively suppressed only when the dose of anti-Rh immunoglobulin is sufficient for ER clearance within 5-8 days [5]. Anti-Rh are virtually unavailable, and we investigated the possibility of using monoclonal antibodies (MAb) and established criteria for their evaluation.

Hematology Research Center, Russian Academy of Medical Sciences; Gematolog Company, Moscow. ***Address for correspondence:*** nolov@blood.ru. Olovnikova N. I.

In vitro capacity of anti-Rh MAb to bind ER antigenic determinants and lyse ER the antibody-dependent cytolytic reaction do not correlate with their capacity to accelerate clearance of Rh^+-ER from circulation [1]. We studied Rh^+-ER clearance in Rh^- volunteers under the effect of four anti-RhD MAb and the development of anti-Rh immune response. MAb with the same *in vitro* activity considerably differ in their capacity to accelerate the clearance of non-sensitized Rh^+-ER. Rapid clearance of ER from circulation not always prevented anti-Rh immune response.

MATERIALS AND METHODS

Four IgG1 MAb (G-7, G-12, G-17, and G-48) produced by heterohybridomas were isolated and purified by affinity chromatography [1] and tested routinely. The concentration of MAb was evaluated by measuring protein content according to Bradford and by the titer of anti-Rh antibodies (WHO standard). The fol-

0007-4888/00/0001-66$25.00

lowing polyclonal antibodies (PAb) were used: anti-Rh immunoglobulin for intravenous injections (LFB, lot 54050091) and anti-Rh immunoglobulin for intramuscular injections (SPK GMU, Russia, lot 061197).

Defrosted washed AB0-identified Rh^+-ER (5 or 15 ml) were intravenously injected to Rh^--recipients. Blood was collected after 10-15 min and the test antibodies were injected (80-120 μg per ml ER suspension). Two groups of recipients received antibodies 1 day before ER. Blood samples were collected after 1, 3, and 7 days, in some recipients after 14 days or 1-3 months. The content of Rh^+-ER in the blood was evaluated by indirect immunofluorescence and flow cytometry on EPICS-C device (Coultronics). ER were stained with G-17 MAb and then with FITC-labeled Fab-fragments of goat anti-human IgG (Jackson IR Lab.). Blood collected before injection of Rh^+-ER served as the negative control. The content of Rh^+-ER in the blood before injection of anti-Rh antibodies was taken as 100%. The number of anti-RhD molecules *in vivo* bound to Rh^+-ER and the maximal saturation of various ER phenotypes with MAb was calculated by the formula:

$$M_{MAb}=M_{PAb}\times 10^{0.01172(Ch2-Ch1)},$$

where M_{PAb} is the known number of Rh determinants on Rh^+-ER detected by PAb [9] and Ch2 and Ch1 are mean values of fluorescence channels on 256-channel histograms for positive PAb and MAb peaks, respectively. Anti-Rh PAb recognized 12,200 (9900-14600) sites on DCe/dce ER and 27,000 (23000-31000) sites on DCe/DcE ER [9]. These values were used to estimate the number of binding sites for MAb.

Immune anti-Rh antibodies in the sera were titered by indirect Coombs' test using a standard ER panel.

RESULTS

The number of antigenic determinants detected *in vitro* by different antigens on the surface of Rh^+-ER with phenotypes DCe/dce and DCe/DcE is shown in Table 1. G-17 MAb was not inferior to PAb, while other MAb detected 1.25-2.31 times less RhD epitopes (Table 1). Only for G-17 MAb *in vivo* and *ex vivo* binding with ER was similar. Other MAb bound no more than 30% antigenic determinants under conditions of excess of antibodies in the serum (data not presented).

The kinetics of Rh^+-ER clearance from circulation after injection of MAb is presented on Fig. 1. Clearance of Rh^+-ER under the effect of anti-Rh PAb served as the control (Fig. 1). MAb differed by their capacity to accelerate ER clearance, the rate of clearance did not directly depend on ER saturation with antibodies. G-17 MAb did not accelerate ER clearance in 4 of 6 volunteers (Fig. 1, *a*), and sensitized ER were detected in the blood after 1-3 months, *i. e.*, their half-life (60 days) corresponded to that of native ER [10]. G-7 and G-12 MAb were most active (Fig. 1, *b, c*), their 1:1 mixture ensured complete clearance of ER within 3 days (Fig. 1, *c*). G-48 MAb accelerated ER clearance, but 20-50% Rh^+-ER were still present in the blood after 2 weeks (Fig. 1, *d*). Complete clearance within 2-6 days was observed in volunteers injected with antibodies 1 day before ER (Fig. 1, *e*). This experimental scheme gave similar results with BRAD-5 and BRAD-3 anti-RhD MAb [7]. Apparently, the interaction of preinjected antibodies with washed ER facilitated their absorption and ensured high density of anti-Rh on ER, which promoted their complete clearance. However, this artificial situation does not characterize true activity of antibodies and cannot be realized under clinical conditions, when immunoglobulin is always injected after penetration of fetal ER into maternal circulation.

TABLE 1. Number of Antigenic Determinants Detectable by MAb and PAb on Rh-Homo- and Heterozygotic Erythrocytes

Antibodies/ ER	PAb	MAb			
		G-7	G-12	G-17	G-48
DCe/DcE	27,000	21,600	21,200	29,100	14,700
DCe/dce	12,200	8000	7300	11,600	5300

Intramuscular injection of PAb and MAb accelerated the clearance of ER but with a 1-day delay (Fig. 1, *f*) due to later entry of antibodies into circulation and 2-fold lower concentration in the blood. Similar results were reported previously [4].

Different MAb differently affected anti-Rh response (Table 2). After single injection of 5-15 ml Rh^+-ER to Rh^- volunteers, antibodies were detected in 50% subjects [2,8,12]. The incidence of immunization was lower in recipients injected with G-17: immune antibodies were detected in 1 of 6 volunteers (16%). No response was observed in recipients Nos. 5-8, in whom ER clearance was not accelerated. These results remind previously declined hypothesis that direct blockade of antigen by antibodies can abolish immune response [2,11]. After injection of G-12 MAb in a dose of 1200-1800 μg/15 ml Rh^+-ER (recipients Nos. 9-11, 18, and 19), antibodies were detected in only 1 recipient (20%), but 4 of 5 volunteers (80%) injected with 600 μg G-12 MAb and 5 ml Rh^+-ER (recipients 20, 21, and 25-27) produced anti-Rh antibodies after 4-5 months. Hence, the effect of G-12 MAb depends on their final concentration in the serum, but not on the dose of Rh^+-ER per ml, in contrast to PAb, whose preventive dose is 20 μg/ml ER.

Anti-RhD PAb in low doses are known to stimulate the immune response [11]. It seems that we observed the same effect with G-12 MAb. PAb in a dose of 100-300 μg prevent [3] and in a dose of 10 μg stimulate the immune response. For G-12 MAb the preventive dose was 1200-1800 μg, while 600 μg stimulated the response, though the volume of injected Rh^+-ER was proportionally decreased. After injections of G-48 and G-7 MAb immunization was noted in 75 and 71% subjects, respectively. In contrast to G-12 MAb,

TABLE 2. Detection of Immune Anti-Rh Antibodies in Rh^- Volunteers at Various Terms after Injection of Rh^+ ER and MAb

Injected antibodies, route and dose	Phenotype and dose of injected Rh^+ER	No. of volunteer	Period after injection, months**	Titer of anti-Rh antibodies	
				direct IgM	Coombs' test
G-48, 1200 μg intravenously	DcE/dce, 15 ml	1	3	—	1:32
		2	4	—	1:32
		3	6	—	—
		4	3	1:2	1:16
G-17, 1200 μg intravenously	DCe/dce, 15 ml	5	6	—	—
		6	6	—	—
		7	6	—	—
		8	5.5	—	—
		36	4	—	1:4
		37	4	—	—
G-12, 1200 μg intravenously	DCe/dce, 15 ml	9	6	—	—
		10	6	1	1:16
		11	6	—	—
G-12+G-48, 600+600 μg intravenously	DCe/DcE, 15 ml	12	5	1:4	1:32
		13	6	—	—
		14	3	1:2	1:16
G-7*, 1200 μg intravenously	DCe/DCe, 15 ml	15	2	1:2	1:16
		16	1.5	1:16	1:16
		17	6	—	—
G-12*, 1800 μg intravenously	DCe/DCe, 15 ml	18	6	—	—
		19	6	—	—
G-12, 600 μg intravenously	DCe/DcE, 5 ml	20	5.5	—	1:32
		21	4	1	1:2
	DCe/DCe, 5 ml	25	4	1:8	1:2
		26	6	—	—
		27	5	1	1:16
G-7, 600 μg intravenously	DCe/DcE, 5 ml	22	2	—	1:32
		23	5	—	—
		24	2	1:8	—
G-7+G-12, 300+300 μg intravenously	DCe/DcE, 5 ml	28	1	1:16	1:16
		29	2	1:16	1:16
		31	1.5	1:64	1:32
G-12, 600 μg intramuscularly	DCe/DCe, 5 ml	32	5.5	—	—
		33	1.5	—	1:8
PAb, 200 μg intramuscularly	DCe/DCe, 5 ml	34	5	—	—
		35	4.5	—	—

Note. *MAb were injected 1 day before ER; ** time when immune antibodies were detected for the first time; if no antibodies were detected, the time of the last analysis is shown.

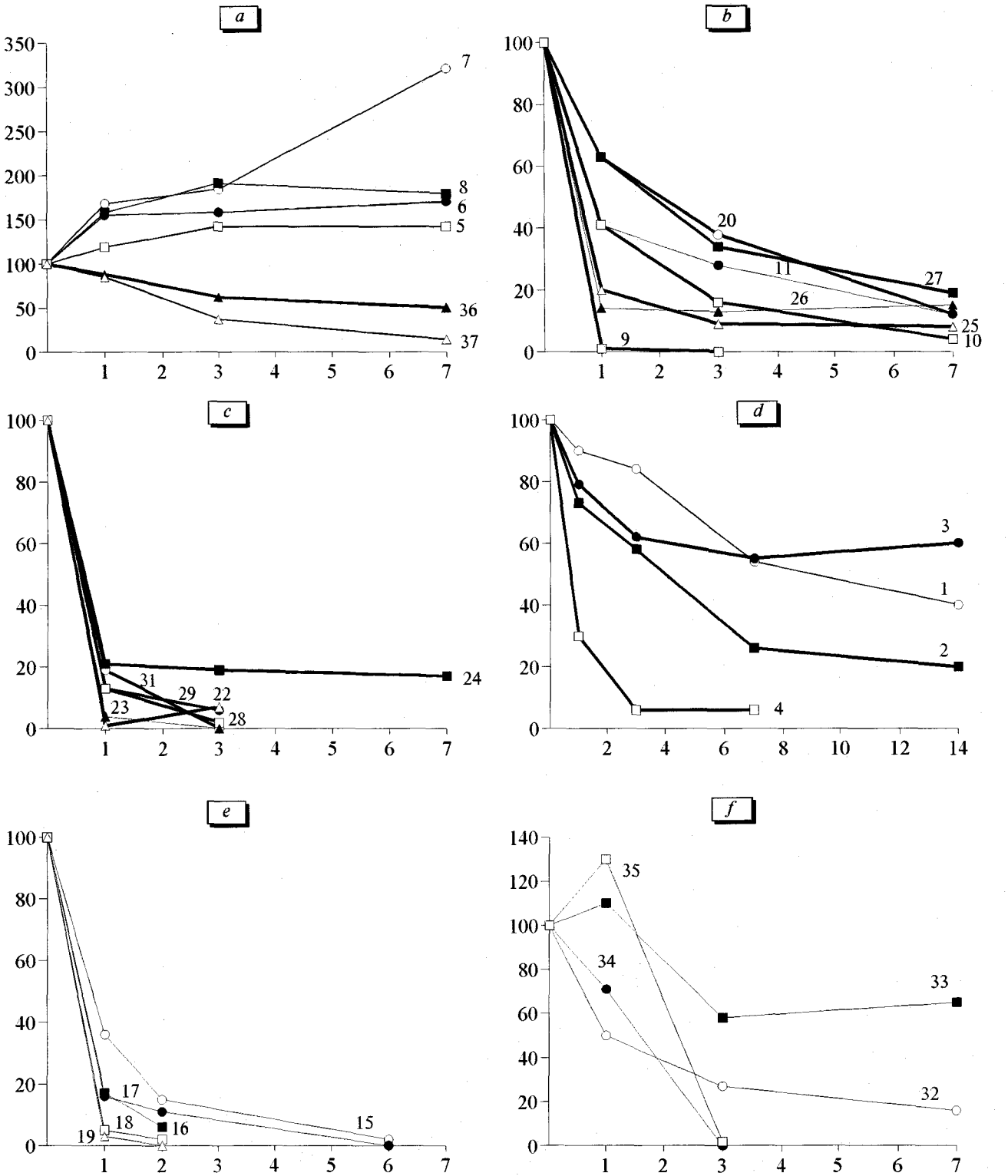

Fig. 1. Clearance of rhesus-positive erythrocytes (Rh^+ ER) from circulation of rhesus-negative recipients under the effect of mono- and polyclonal anti-rhesus antibodies. Ordinates: percentage of circulating Rh^+ ER (Rh^+ ER content in the blood before injection of the antibodies is taken as 100%); abscissa: time after injection of antibodies, days. Bold lines: ER clearance in volunteers who later developed anti-rhesus antibodies. *a*) G-17 MAb, 1200 μg+ER, 15 ml; *b*) G-12 MAb, 600 μg+ER, 5 ml (for recipients Nos. 20, 21, 25-27); G-12, 1200 μg+ER, 15 ml (for recipients Nos. 9-11); *c*) G-7 MAb, 600 μg+ER, 5 ml (for volunteers Nos. 22-24); G-7+G-12, 600 μg+ER, 5 ml (for volunteers Nos. 29-31); *d*) G-48 MAb, 1200 μg+ER, 15 ml; *e*) G-7 MAb, 1200 μg+ER, 15 ml (for volunteers Nos. 15-17); G-12, 1800 μg+ER, 15 ml (for volunteers Nos. 18 and 19) before ER; *f*) G-12 MAb, 600 μg+ER, 15 ml (for volunteers Nos. 32 and 33); polyclonal antibodies intramuscularly, 1000 IU+ER (for volunteers Nos. 34 and 35). Figures near curves are numbers of volunteers.

the effect of G-7 MAb was dose-independent. The highest in our study titers of anti-Rh IgM and IgG 1-2 months after immunization were observed in subjects injected with a mixture of G-7 and G-12 MAb, causing the most rapid clearance of ER. It is surprising that in absolutely different groups answering by attenuation or stimulation of immune response, ER clearance was sharply accelerated in all cases.

Previously we demonstrated different behavior of anti-Rh MAb *in vitro* in antibody-dependent cytolysis and *in vivo* during acceleration of the clearance of autologous and allogenic Rh^+-ER [1]. Our findings indicate that rapid clearance of Rh^+-ER is the decisive condition for preventing sensitization, while blockade of immune response cannot be explained only by destruction of the antigen in the spleen. It has been recently shown that binding of antibody Fc-fragments to Fc-receptors on effector cells can stimulate the activating or inhibitory modulus of the antigen. These modules represent thyrosine-rich cytoplasmic sequences of the Fc-receptor, responsible for stimulation or blocking of the immune response [6]. Activation of this or that modulus depends on the conformation and number of antibodies on the cell membrane, their distribution, *etc.* Different anti-RhD antibodies can be specifically recognized by this system irrespective of their *in vitro* activity and effects on ER clearance. Further studies of G-12 and G-7 MAb will help to elucidate the mechanisms of blockade of the immune response.

The study was supported by the Russian Foundation for Basic Research (grant No. 96-04-48156). The authors are grateful to E. Yu. Sadovnikov, Cand. Biol. Sci., for fruitful discussion of the results.

REFERENCES

1. N. I. Olovnikova, E. V. Belkina, T. L. Nikolaeva, and I. L. Chertkov, *Byull. Eksp. Biol. Med.*, **125**, No. 1, 71-74 (1998).
2. J. M. Bowman, *Transfus. Med. Rev.*, **2**, No. 3, 129-150 (1988).
3. M. Conteras, *Current Problems of Transfusion Medicine in Clinical Practice*, St. Petersburg (1993), pp. 143-146.
4. M. J. Goodrick, B. M. Kumpel, D. H. Pamphilon, *et al.*, *Clin. Exp. Immunol.*, **98**, No. 1, 17-20 (1994).
5. N. C. Hughes-Jones, *Br. J. Haematol.*, **70**, No. 3, 263-265 (1988).
6. N. Isakov, *Immunol. Res.*, **16**, No. 1, 85-100 (1997).
7. B. M. Kumpel, M. J. Goodrick, D. H. Pamphilon, *et al.*, *Blood*, **86**, No. 5, 1701-1709 (1995).
8. P. L. Mollison, *Hemolytic Disease of the Newborn*, Ed. G. Garratty, Arlington (1984), pp. 1-32.
9. P. L. Mollison, C. P. Engelfriet, and M. Contreras, *Blood Transfusion in Clinical Medicine*, Oxford (1997), p. 154.
10. P. L. Mollison, C. P. Engelfriet, and M. Contreras, *Ibid.*, pp. 279-282.
11. W. Pollack, *Hemolytic Disease of the Newborn*, Ed. G. Garratty, Arlington (1984), pp. 53-65.
12. W. Pollack, W. Q. Ascari, R. J. Kochesky, *et al.*, *Transfusion*, **11**, No. 6, 333-339 (1971).