EFFECT OF ANTIBODIES AGAINST γ-GLOBULIN ON CYTOTOXIC ACTIVITY OF IMMUNE LYMPHOCYTES IN AN ALLOGENIC CELL CULTURE

B. D. Brondz, E. S. Ievleva, and G. N. Vornakova

UDC 612.112.94:612.017.1

Rabbit antibodies against mouse γ-Globulin neutralized humoral isocytotoxins of inbred mice but had no effect on the cytotoxic activity of immune lymphocytes in vitro. γ-Globulin was found on the surface of 14-30% of lymphocytes. The results indicate that the activity of immune lymphocytes is not due to secreted antibodies.

* * *

It has been shown experimentally during recent years that the cytotoxic action of immune lymphocytes on allogenic cells cultivated in vitro is not due to humoral antibodies synthesized or adsorbed by lymphocytes [3-5, 14, 20]. Yet at the same time, γ-globulins [16, 19] or their fragments [17] have been found by various methods in the membrane of small lymphocytes. By a combination of Jerne's method with electron microscopy, lymphocytes synthesizing antibodies have been detected [11].

In the present investigation the effect of antibodies against γ-globulin on the cytotoxic activity of lymphocytes and humoral antibodies was studied.

EXPERIMENTAL METHOD

Experiments were carried out on mice of inbred lines C57BL/10Sn (H-2b), B10 · D2(H-2^d), and C3H/DiSn (H-2k) aged 9-12 weeks.

The tumors were polymorphocellular sarcomas induced by methylcholanthrene in mice of lines C57BL/10Sn and B10 · D2. Suspensions of tumor cells were obtained by trypsinization.

Mouse (MGG) and Rabbit (RGG) γ-globulins were isolated from serum by preparative electrophoresis in agar [6].

Rabbits were hyperimmunized with MGG by 7-8 intramuscular injections each of 25-40 mg protein. Antibodies against MGG were detected by precipitation in agar using a standard test system [7]. The titer of antibodies against MGG in the rabbit immune serum (RIS) was 1 : 128, and in rabbit immune γ-globulin (RIG) it was 1 : 64. Immunoelectrophoresis in agar showed that RIS against MGG reacted not only with MGG, but also with a mouse α- and β-globulins. Absorption of RIS, RIG, normal rabbit serum (RNS), and normal rabbit γ-globulin (RNG) was carried out for 1 h at 37° and for 30 min at 4° with 5-times washed mouse erythrocytes and lymphocytes. Each portion of the preparation was absorbed twice: 0.5 vol. of erythrocyte sediment and 0.25 vol. of lymphocyte sediment. The absorbed preparations were sterilized by passage through a Seitz filter and kept at −20°. The protein content in the absorbed RIG and RNG was 6.7-7.2 mg/ml.

To obtain humoral isoantibodies mice were immunized subcutaneously with $2 \cdot 10^7$ living washed allogenic lymphocytes 5 times at intervals of 1-2 weeks. The serum was kept at −20°. Isoantibodies were determined by means of the cytotoxic reaction in vitro [10] in our modification [2].

Immune lymphocytes were obtained from the regional lymph glands of mice 8 days after a single immunization with allogenic tumor [1]. The scheme of the experiment to study the cytotoxic effect of immune

Department of Virology and Immunology of Tumors, N. F. Gamaleye Institute of Epidemiology and Microbiology, Moscow (Presented by Active Member of the Academy of Medical Sciences of the USSR G. V. Vygodchikov). Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 66, No. 12, pp. 73-77, December, 1968. Original article submitted October, 1967.

TABLE 1. Neutralization of Humoral Mouse Isoantibodies by Rabbit Antibodies against Mouse γ-Globulin

Dilutions of mouse antiserum[1]	Cytotoxic index after incubation of isoantiserum with: PS	RIS dilutions 1:2	RIS 1:4	RIS 1:8	RNS 1:2	RIG 1:2,5	RIG 1:5	RIG 1:10	RIG 1:20	RIG 1:40	RNG 1:2
1:5	0,95	0,14	0,70	0,73	0,85						
1:10	0,84	0			0,72						
1:10	1,0					0,94	0,99	0,98	1,00	1,00	1,00
1:40	0,95					0	0	0,33	0,79	0,91	
1:80	0,92					0	0	0,01	0,26	0,46	0,90
1:160	0,21					0	0	0	0,01	0,08	0,20
1:320	0,13										
1:640	0,02										
Normal mouse serum[2]	(82,7)	0			0,02						
	(84,9)	0			0,07						
	(86,0)	0				0			0		0
	(87,3)	0									

[1]B10 · D2 anti-C57BL/10 antiserum, cytotoxic titer 1:160.
[2]B10 · D2 serum, percentage of living C57BL/10 cells in different experiments after incubation with normal serum shown in parentheses.
Legend: PS physiological saline, RIS rabbit immune serum against mouse globulin, RNS normal rabbit serum, RIG rabbit immune γ-globulin against mouse globulin, RNG normal rabbit γ-globulin. The RIS, RNS, RIG, and RNG were adsorbed by mouse cells.

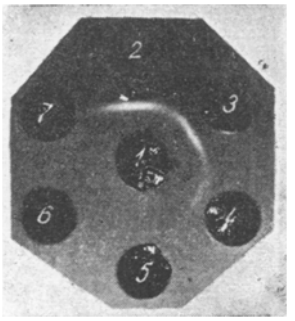

Fig. 1

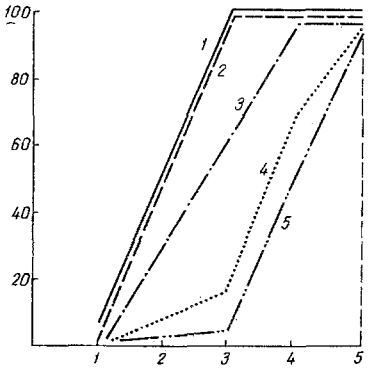

Fig. 2

Fig. 1. Precipitation of rabbit immune γ-globulin (RIG) adsorbed by mouse cells with mouse γ-globulin (MGG) in agar. 1 and 2) Test system for MGG; 1) MGG 50 mg/ml, 2) rabbit antiserum against MGG in optimally reacting dilution; 3-7) double dilutions of RIG from 1:16 (3) to 1:256 (7).

Fig. 2. Neutralization of mouse isoantibodies by rabbit immune γ-globulin (RIG) against mouse γ-globulin. Abscissa, logarithms of dilutions of anti-C57BL/10 antiserum of B10 · D2 mice, ordinate, neutralization of isoantibodies in percent: (a-b) ×100 where a is the cytotoxid index of the antiserum after incubation with physiological saline; b is the same after incubation with dilutions of RIG. Dilutions of RIG: 1) 1:2.5, 2) 1:5, 3) 1:10, 4) 1:20. Cytotoxic titer of antiserum shown by broken line.

TABLE 2. Cytotoxic Effect in Vitro of Lymphocytes Treated with Rabbit Antibodies against Mouse γ-Globulin

Type of lymphocytes	Dose of lymphocytes (in millions)	Expt. No.	No. of living macrophages (× 10³) after incubation with lymphocytes			% of effect of immune lymphocytes			% of neutralization of effect in immune lymphocytes by RIS or RIG
			intact	treated with[1]		intact	treated with		
				RIS or RIG	RNS or RNG		RIS or RIG	RNS or RNG	
1	2	3	4	5	6	7	8	9	10
Normal	20	1	71,6±16,1	67,1±12,7	87,6±6,3				
	20	2	65,9±4,7	51,4±4,8	52,8±7,3				
	8	2	59,4±7,4	56,8±6,6	59,4±8,0				
	10	3	53,6±6,1	—	—				
	20	4[4]	112 ±20,0	—	—				
	20	5[4]	95 ±3,5	93,6±12,8	—				
	10	6[4]	101 ±7,2	104±4,4	—				
Immune	20	1	0	0	0	100	100	101	0
	20	2	<3	<3	<3	>95,5	>94,1	>94,3	0,8
	8	2	25,3±5,1	29,4±11,6	38,1±5,5	57,4	48,4	35,9	0
	10	3	11,3±4,4	0	7,6±8,0	78,9	100	85,8	0
	20	4[4]	15,1±2,9	17,6±7,9	7,0±7,5	86,5	84,3	93,7	6,9
	10	4[4]	46,2±8,1	52,8±4,9	—	58,7	52,9	—	9,9
	20	5[4]	5,9±3,3	7,4±5,8	11,4±3,4	93,7	92,1	88	0
	7,5	5[4]	18,8±6,1	23,4±7,1	13,4±1,8	80,2	75,0	85,9	9,6
	10	6[4]	61±7,1	42±14,9	—	39,6	59,6	—	0

[1]Abbreviations as in Table 1. RIS or RNS used in experiments Nos. 1, 2, and 3, RIS and RNG in experiments Nos. 4, 5, and 6.

[2]$\frac{a-b}{a} \times 100$, where a and b are the numbers of macrophages after incubation with normal (a) and immune (b) lymphocytes.

[3]Either the value in column 7 or the mean of the values in columns 7 and 9 is taken as 100%.

[4]Rabbit preparations added to the medium along with lymphocytes. In experiments Nos. 1, 2, 3, 5, and 6 the immune lymphocytes were B10 · D2 anti-C57BL/10, and in experiment No. 4 they were C3H anti-B10 · D2.

lymphocytes on allogenic peritoneal macrophages growing in monolayer cultures has been described previously [1, 3, 4].

To neutralize the serum isoantibodies, different dilutions of isoantisera were incubated for 1 h at 37° with the same volume (0.05 ml) of absorbed RIS (or RIG) and in control tests with absorbed RNS, RNG, or physiological saline. The precipitate thus formed was not removed because its presence in the medium had no nonspecific cytotoxic action on the cells.

In the experiments to study neutralization of lymphocytes, $40 \cdot 10^6$–$100 \cdot 10^6$ living cells were mixed with absorbed preparations: 0.35 ml of whole RIS or 2.5 ml of RIG, diluted 1 : 5–1 : 10. Lymphocytes in the control tubes were treated with whole RIS, diluted RIG, or culture medium. After incubation for 40–60 min at 37° the supernatant was either removed by a single centrifugation (in the experiment with RIS) or it was added to the culture along with the lymphocytes (in experiments with RIG).

Absorption of rabbit antibodies against MGG on the surface of living unfixed lymphocytes was detected by immunofluorescence [13]. For this purpose a portion of the treated cells was washed and incubated with eluate of pure antibodies against rabbit γ-globulin. The eluate was isolated from ass serum on an immunosorbent, conjugated with fluorescein isothiocyanate, and absorbed with powdered liver [8].

EXPERIMENTAL RESULTS

Preliminary experiments showed that unabsorbed rabbit sera and globulins contained normal antibodies against mouse lymphocytes, leading to agglutination of the lymphocytes and a cytotoxic effect in the presence of complement (RNS 1 : 2 killed about 30% of the cells, 1 : 20 about 10%). After removal of normal

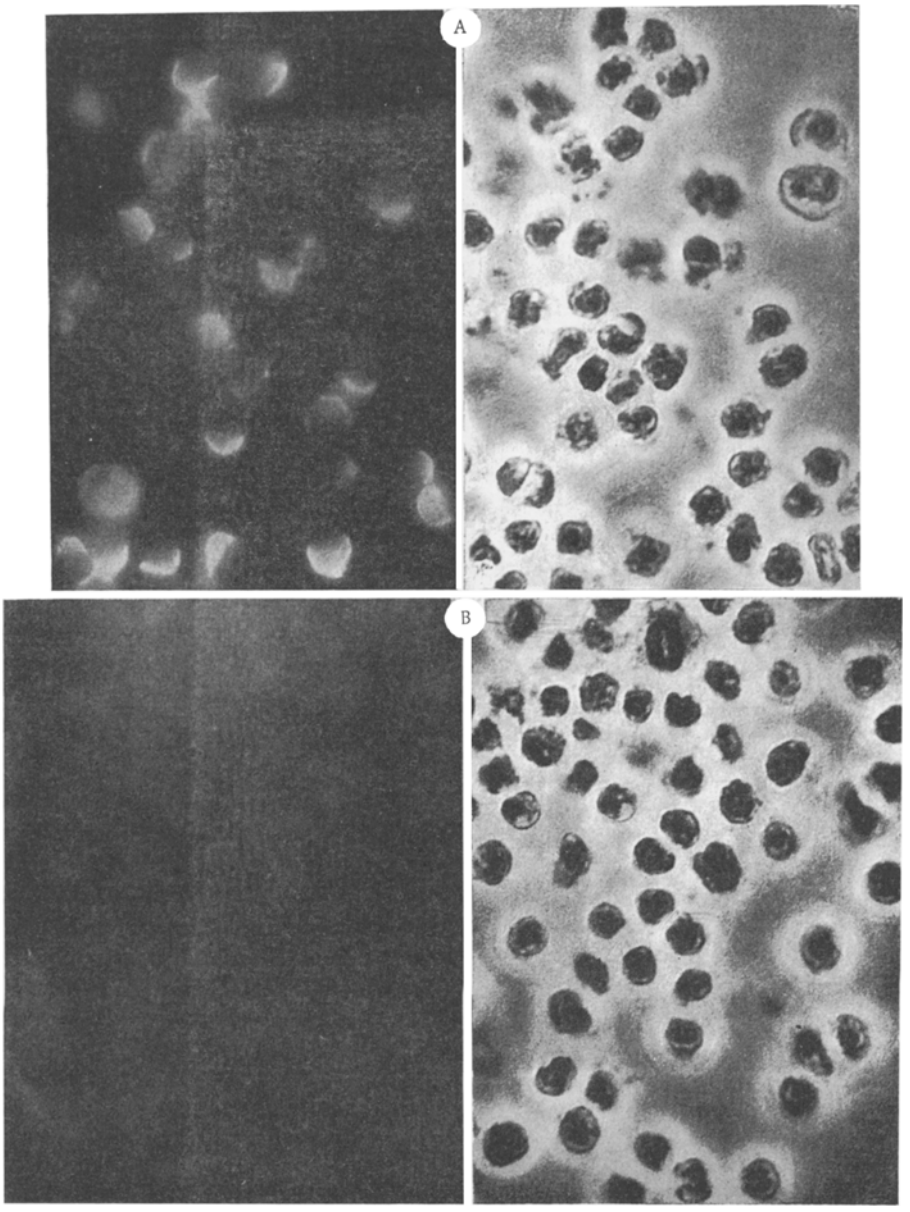

Fig. 3. Unfixed preparations of immune B10 · D2 anti-C57BL/10 lymphocytes treated successively with anti-MGG RIS (A) or RNS (B) and with labeled ass antibodies against rabbit γ-globulin. Fluorescence on the left, phase contrast on the right.

antibodies by absorption with mouse cells the RIS and RIG still contained antibodies against MGG in a titer of 1 : 64-1 : 32 (Fig. 1), whereas their cytotoxic activity against mouse lymphocytes had disappeared (Table 1).

Table 1 shows that the absorbed RIS and RIG neutralized the humoral anti-C57BL/10 isocytotoxins of B10 · D2 mice whereas neither RNS nor RNG had any effect on the results of the cytotoxic tests. The RIS activity exceeded the RIG activity. The neutralizing effect of RIG dilutions was dependent on the dilutions of the mouse isoantiserum (Fig. 2). Dilutions 1 : 5 and 1 : 10 of RIG, neutralizing isoantiserum diluted 1 : 40 and 1 : 80 respectively were used in the subsequent experiments.

The cytotoxic activity of lymphocytes treated with absorbed rabbit preparations was tested in 6 experiments (Table 2). Intact immune lymphocytes destroyed 39.6-100% of the cells of the culture (column 7)

depending on the number of lymphocytes added to the culture (from 7.5 to 20 million). Treatment of the immune lymphocytes with RIS or RIG did not reduce the cytotoxic effect (columns 8 and 9). Its reduction to 9.9% in these cases (column 10) is not statistically significant. Normal lymphocytes treated with rabbit preparations did not produce a significant cytotoxic effect.

The results of the immunofluorescence experiment showed that RIS and RIG were adsorbed on the surface of the lymphocytes causing fluorescence of the cell membrane in the form of a semicircle or a smaller segment (Fig. 3A). If whole RIS was used fluorescence was found in 30% (241 of 797) of immune and 20% (186 of 618) of normal B10 · D2 lymphocytes. RIG in a dilution of 1 : 10 caused fluorescence of 14.1% (121 of 736) of immune and 13.7% (93 of 585) of normal B10 · D2 lymphocytes. As a result of treatment of the lymphocytes with RNS or RNG (control) no fluorescent cells were found (Fig. 3B).

Rabbit antibodies against MGG thus neutralized the humoral isoantibodies but had no effect on activity of the immune lymphocytes in tissue culture. The absence of action on lymphocytes was evidently unconnected with the quantitative ratios between antibodies against MGG and lymphocytes: a decrease in the number of lymphocytes by 2.5 times while the antibody content remained constant did not lead to inhibition of lymphocyte activity. The results likewise cannot be explained by a deficiency of antibodies in the culture medium during interaction between lymphocytes and target cells: RIG was added to the medium together with lymphocytes in a concentration neutralizing the humoral cytotoxins. These results agree with those obtained previously in vivo [18], and they show that the cytotoxic effect of immune lymphocytes is not due to humoral antibodies secreted into the medium and acting in the immediate vicinity of the lymphocytes. The possibility is not ruled out that the lymphocytes may "inject" high concentrations of antibodies into the target cell when direct contact takes place between the cell surfaces, and anti-γ-globulin cannot interfere with this process. However, this explanation is unlikely because the chief active cell in the system studied is the small lymphocyte [12], containing no ergastoplasm and incapable of secreting large quantities of protein extracellularly.

The immunofluorescence method in this investigation confirmed the presence of γ-globulin on the surface of 14-30% of lymphocytes. The role of this surface γ-globulin in the cytotoxic activity of the lymphocytes is unclear. It is possible that antibodies against MGG, although blocking the antigenic area of the antibody molecule, cannot neutralize (or eliminate from the reaction) the active center of this antibody if it is not in solution but is fixed on the cell surface.

However, this explanation does not agree with the experimental results: the immunoadhesion reaction caused by antibodies fixed on the cell surface was inhibited by antibodies against the corresponding γ-globulin [9].

The rabbit antibodies used in this investigation were active mainly against mouse IgG globulin. However, there is experimental evidence that the activity of immune lymphocytes may be associated with IgA globulin in rats [21], and guinea pigs [15]. It may be considered that the approach used in the present investigation may be useful for the further study of this problem*.

LITERATURE CITED

1. B. D. Brondz, Vopr. Onkol., No. 8, 64 (1964).
2. B. D. Brondz, Byull. Éksperim. Biol. i Med., No. 5, 64 (1964).
3. B. D. Brondz, Folia Biol. (Praha), 10, 164 (1964).
4. B. D. Brondz, Transplantation, 3, 356 (1965).
5. B. D. Brondz and L. M. Bartova, Folia Biol. (Praha), 12, 431 (1966).
6. L. A. Zil'ber and G. I. Abelev, The Virology and Immunology of Cancer [in Russian], Moscow (1962), p. 383.
7. N. I. Khramkova and G. I. Abelev, Neoplasma (Bratisl.), 10, 121 (1963).
8. N. V. Engel'gardt, Byull. Éksperim. Biol. i Med., No. 1, 67 (1964).
9. G. Biozzi, C. Stiffel, D. Monton, et al., Ann. Inst. Pasteur, 110, Suppl., 7 (1966).
10. P. A. Gorer and P. O'Gorman, Transplant Bull., 3, 142 (1956).
11. T. N. Harris, K. Hummeler, and S. Harris, J. Exp. Med., 123, 161 (1966).
12. D. S. Janowsky, W. Rosenau, and H. D. Moon, Proc. Soc. Exp. Biol. (New York), 115, 77 (1964).

* The authors are grateful to N. V. Engel'gardt, N. I. Kuprina, and T. V. Beloshapkina for supplying the rabbit immune sera, γ-globulins, and eluate of ass anti-rabbit antibodies and for determining their activity.

13. G. Möller, J. Exp. Med., 114, 415 (1961).
14. W. Rosenau and H. D. Moon, J. Immunol., 96, 80 (1966).
15. U. S. E. Rothman, Acta Soc. Med. Upsalien., 71, 109 (1966).
16. S. Sell, J. Exp. Med., 125, 393 (1967).
17. S. Sell, J. Immunol., 98, 786 (1967).
18. A. M. Silverstein and K. L. Kraner, Transplantation, 3, 535 (1965).
19. R. Van Furth, H. R. E. Schuit, and W. Hijmans, Immunology, 11, 29 (1966).
20. D. B. Wilson, J. Exp. Med., 122, 143 (1965).
21. G. F. Winkler and B. G. Arnason, Science, 153, 75 (1966).