Optimal Detection of Serum Antipaternal Antileukocytic Antibodies after Injection of Allogenic Lymphocytes in Women with Habitual Abortions

L. V. Krechetova, M. A. Nikolaeva, L. V. Van'ko, M. M. Ziganshina, E. L. Golubeva, E. O. Stepanova, G. T. Sukhikh

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 5, pp. 684-688, May, 2012
Original article submitted February 6, 2011

The level of antipaternal antileukocytic antibodies detected by flow cytometry is a parameter reflecting the efficiency of alloimmunization of women with reproductive disorders during preparation to pregnancy. The results of evaluation of antipaternal antileukocytic antibodies by two modifications of the method are presented. The optimal method for detection of antipaternal antileukocytic antibodies after immunocytotherapy is selected.

Key Words: *alloimmunization; antipaternal antileukocytic antibodies; flow cytometry*

Poor allogenic stimulation of the maternal immune system with fetal paternal antigens is assumed to be a cause leading to the development of immune disorders of gestation at its different stages. For example, coincidence of HLA alleles in the parents reducing mutual allogenic stimulation may be an alloimmune factor leading to disorders in the normal course of pregnancy [10,11]. For this reason, immunocytotherapy (ICT; immunization of women by partner's or donor lymphocytes) is now often recommended for preparation of women to pregnancy in couples with HLA compatibility [1,3]. The theoretical basis of this approach is effective use in transplantology of intravenous injection of donor antigens, stimulating the recipient tolerance of donor antigens [5,14].

In obstetrics and gynecology injection of allogenic lymphocytes for additional immunization of women with reproductive function disorders of alloimmune origin during preparation to pregnancy promotes the formation of the immune status essential for implantation, stimulation of placenta development, and support of normal development of the embryo [3,4]. One of the parameters reflecting the development of specific response of the female lymphocytes to alloantigens after ICT is the level of antipaternal antileukocytic antibodies (APAB) in the female serum.

The prototype of APAB detection method is known in transplantology as the cross cytotoxicity method, consisting in detection of recipient serum antibodies (AB) binding to donor lymphocyte surface antigenic determinants. Use of flow cytometry (FCM) for identification of these AB improved the sensitivity of the method and made it a routine clinical method in transplantology [13]. Several modifications of FCM are known – with different duration and temperatures for recipient serum incubation with donor cells and different dilutions of test sera and cell concentrations [15]. The modification suggested by M. Ormerod is recommended as the golden standard for screening for cross-reacting AB in transplantology [12].

The method for detection of APAB by FCM consists in evaluation of binding of FITC-labeled AB to human IgG to the antibodies on the surface of partner's lymphocytes. The method was used for evaluating the efficiency of immunization of women with repeating spontaneous abortions [7,8]. One of the modifications was suggested by T. Maruyama [9]. However, we used it for evaluating the efficiency of immunization of

V. I. Kulakov Center of Obstetrics, Gynecology, and Perinatology, Ministry of Health and Social Development of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** k_l_v@mail.ru. L. V. Krechetova

women with reproductive disorders and found that rather many patients exhibited no linear time course of APAB levels after a series of ICT procedures.

We compared two methods for APAB detection by flow cytometry (Maruyama, method 1; Ormerod, method 2) and selected the optimal method for monitoring of the efficiency of successive alloimmunizations for correction of immunoregulation of gestation.

MATERIALS AND METHODS

The blood was collected after overnight fasting from the ulnar vein into sterile tubes. Lymphocytes were isolated from the partners' whole blood by density gradient centrifugation (ficoll-verograffin gradient, 1.077 g/ml) [2]. The female sera were obtained after blood clot formation (30 min, ambient temperature) and centrifugation (900*g*, 10 min). The sera were inactivated by heating (30 min, 56°C) before the analysis.

The data obtained by the two methods for APAB evaluation are presented in Table 1. The count of male lymphocytes in the samples was no higher than 10^6/ml. The samples were centrifuged at 900*g* for 10 min between washings. Inactivated pooled AB-serum (Sigma) served as the nonspecific binding control. FITC-labeled antibodies to human immunoglobulin Fc fragment (Sigma) were used. Analysis was carried out on a FACScan flow cytofluorometer (Becton Dickinson).

Sera from 170 women receiving ICT during preparation to pregnancy were analyzed. Of these, APAB were evaluated by method 1 in 82 and by method 2 in 88. Immunocytotherapy was administered at 1-month interval. It consisted in intracutaneous injection of 50×10^6 suspended leukocytes from the partner; APAB were evaluated 3-4 weeks after each ICT procedure (before the next one).

The data were presented as the mean±error in the mean ($M\pm m$). The significance of differences in the mean estimated values was evaluated by two-sample *t* test with different dispersions.

RESULTS

Immunocytotherapy by both methods significantly ($p<0.001$) increased APAB level in women with reproductive disorders (Fig. 1). However, the values obtained by method 2 were significantly higher than those obtained by method 1 ($p<0.001$).

A nonlinear relationship between APAB level and duration of male lymphocytes incubation with the female serum and with the female serum dilution was observed in measurements by method 1 (Figs. 2, 3). This indicated that the conditions of analysis in this modification were not optimal.

The main feature of method 1 was incubation of male mononuclear cells (MNC) and female serum at ambient temperature with subsequent washing of the mixture at 4°C, while in method 2 the male MNC and female serum were incubated at 37°C and then washed at ambient temperature, that is, under more physiological conditions. Manipulations at low temperatures lead to formation of, for example, active oxygen species [6] promoting damage to molecules, for example, impairment of the structure of antigen complexes with AB, leading to their disintegration or desquamation from cell surface. In addition, cold and warm AB have been described, with antigen binding constants determined by the incubation mixture temperature. These factors could be responsible for lower fluorescence of cells recorded by method 1 (Figs. 2, 3) and hence, unstable results of APAB measurements by this method (Fig. 1), which limited its use for evaluation of the effects of successive alloimmunizations.

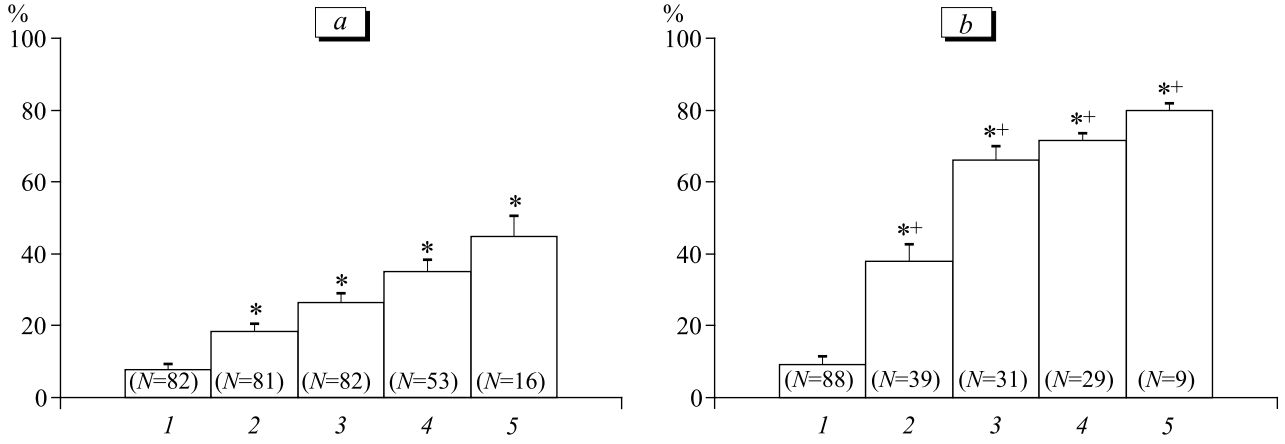

Fig. 1. Measurements of APAB by method 1 (*a*) and method 2 (*b*). *1*) before ICT; *2*) ICT procedure 1; *3*) ICT procedure 2; *4*) ICT procedure 3; *5*) ICT procedure 4. N: number of examined patients. $p<0.001$ in comparison with: *level before ICT, +respective ICT carried out by the other method.

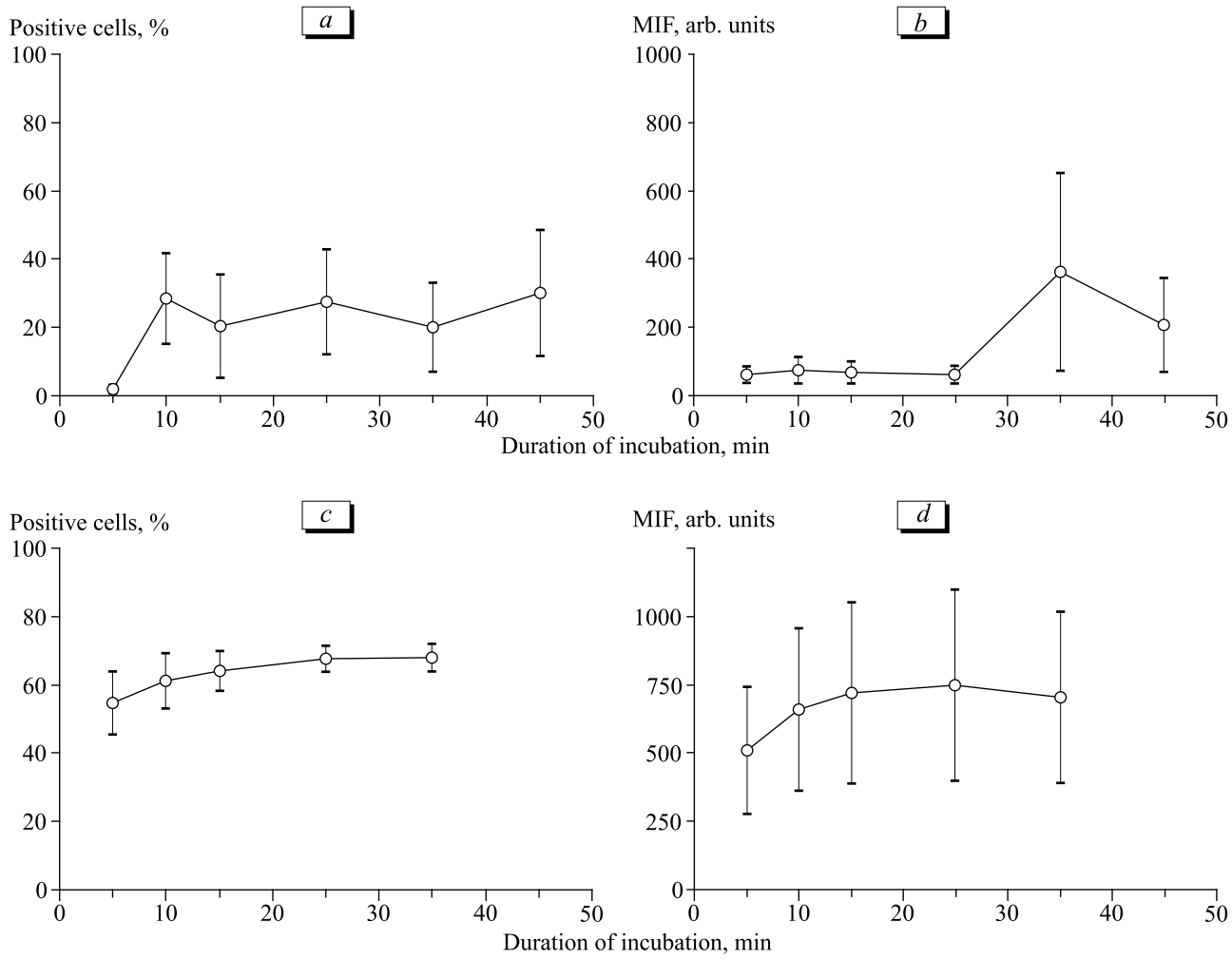

Fig. 2. Relationship between the percentage of ABAP-coated male lymphocytes (*a*, *c*) and mean fluorescence intensity (MIF) of positive cells (*b*, *d*) and the duration of incubation with the female serum evaluated by method 1 (*a*, *b*; *n*=6) and method 2 (*c*, *d*; *n*=4).

TABLE 1. Main Parameters of FCM Variants

Steps	Parameters	Values	
		method 1	method 2
Incubation of male MNC with female serum	Incubation temperature, °C	23	37
	Duration of incubation, min	15	30
	Serum dilution	1:4	1:1
Washing of male MNC from female serum	Washing solution	PS with 0.5% BSA	PS with 1% FCS
	Number of washings	2	3
	Temperature of washing, °C	4	23
Incubation of male MNC with second AB	Duration of incubation, min	15	30
Washing of male MNC from second AB	Washing solution	PS with 0.5% BSA	PS with 1% FCS
	Number of washings	2	1
	Temperature of washing, °C	4	4

Note. PBS: phosphate buffered saline.

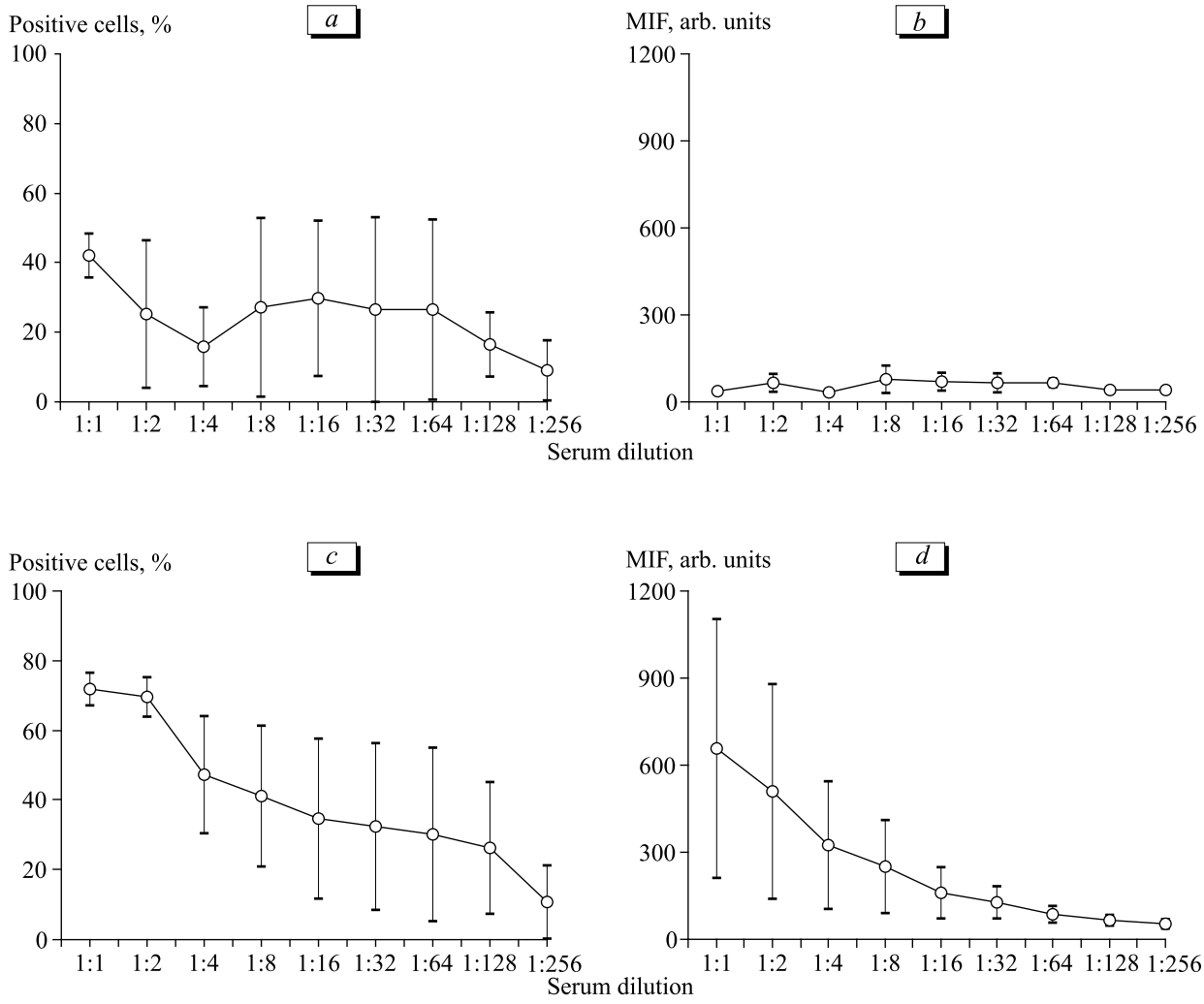

Fig. 3. Relationship between the percentage of ABAP-coated male lymphocytes (*a*, *c*) and mean fluorescence intensity (MIF) of positive cells (*b*, *d*) and the female serum dilution, evaluated by method 1 (*a*, *b*; *n*=4) and method 2 (*c*, *d*; *n*=4).

Hence, measurements of APAB by FCM suggested by M. Ormerod (method 2) proved to be the optimal method for monitoring the efficiency of alloimmunizations for correction of reproductive disorders.

REFERENCES

1. V. I. Govallo and V. M. Sidel'nikova, *Akush. Ginekol.*, No. 12, 25-27 (1983).
2. V. V. Men'shikov, L. N. Delektorskaya, R. P. Zolotnitskaya, *et al.*, *Laboratory Methods of Studies in Clinical Practice* [in Russian], Moscow (1987).
3. V. M. Sidel'nikova, *Habitual Abortions* [in Russian], Moscow (2005).
4. G. T. Sukhikh and L. V. Van'ko, *Immunology of Pregnancy* [in Russian], Moscow (2003).
5. S. Agrawal, R. Kishore, A. Halder, *et al.*, *Hum. Reprod.*, **10**, No. 9, 2280-2284 (1995).
6. S. S. Ali, M. C. Marcondes, H. Bajova, *et al.*, *J. Biol. Chem.*, **285**, No. 42, 32,522-32,528 (2010).
7. S. Chaichian, S. Shoaee, A. Saremi, *et al.*, *Am. J. Reprod. Immunol.*, **57**, No. 3, 169-176 (2007).
8. A. Gilman-Sachs, S. P. Luo, A. E. Beer, and H. D. Beaman, *J. Clin. Lab. Immunol.*, **30**, No. 2, 53-59 (1989).
9. T. Maruyama, T. Makino, T. Sugi, *et al.*, *Am. J. Obstet. Gynecol.*, **168**, No. 5, 1528-1536 (1993).
10. H. S. Nielsen, M. D. Witvliet, R. Steffensen, *et al.*, *J. Reprod. Immunol.*, **87**, Nos. 1-2, 67-73 (2010).
11. C. Ober, T. Hyslop, S. Elias, *et al.*, *Hum. Reprod.*, **13**, No. 1, 33-38 (1998).
12. M. G. Ormerod, *Flow Cytometry: A Practical Approach*, Oxford (2000).
13. S. Riethmuller, S. Ferrari-Lacraz, M. K. Müller, *et al.*, *Transplantation*, **90**, No. 2,160-167 (2010).
14. M. F. Reznikoff-Etievant, I. Durieux, R. Lobet, *et al.*, *Rev. Fr. Transfus. Immunohematol.*, **29**, No. 3, 135-148 (1986).
15. B. K. Shenton, A. E. Bell, A. W. Harmer, *et al.*, *Transplant. Proc.*, **29**, Nos. 1-2, 1454-1455 (1997).