AGGLUTINATION REACTION PROCEDURES FOR NORMAL AND TUMOR TISSUE CELLS AND FOR THE CYTOTOXIC REACTION IN VITRO

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At present there are available data pertaining to the distribution of different antigens in the cell wall: species specific [24], tissue specific [3], H-2 transplantation locus [10, 14, 15] and, apparently, tumor specific. Location of the determinant groups of the transplantation antigens on the cell surface is a factor responsible for cytotoxicity of iso-antibodies [15]. It is entirely possible that all these antigens are insoluble and that the routine serological procedures may prove to be inadequate for their disclosure. In this connection, procedures for reactions such as agglutination of tissue cells and the in-vitro cytotoxic test, should be improved and made more sensitive.

The agglutination reaction of normal and tumor cells by the heterologous antibodies has been used by many investigators [1, 2, 5, 11, 13, 21]. This procedure has been developed in greater detail by Kite and co-workers [13]. We added to it a number of corrections and additions.

Cell suspensions of the solid tumors (carcinoma and sarcoma) is obtained by trypsinization (other methods, such as passage through a metal sieve, homogenization of a tumor for a short period of time, etc., are not suitable, since they lead to destruction of most of the cells). A tumor, after careful removal of the necrotic areas, is cut up with irridectomy scissors, the pieces are washed twice in large volumes of Ringer's solution to remove broken cells, erythrocytes and mucus, they are covered with 0.25% trypsin solution and are left overnight at 4° in flat-bottom 50

TABLE 1. The Use of Different Concentrations of Normal and Tumor Cells of Mice in Agglutination Reaction by Immune Rabbit Sera Against Mouse Tumors

Serum dilution	Immune serum against mouse sarcome SaI						Immune serum against mouse sarcoma MX2								
	sarcoma SaI						lymphocytes from lymph nodes				spleen			liver	kidney
	final concentration of cells per 1 ml in test tubes														
	$2\cdot10^5$	$14\cdot10^5$	10^6	$2\cdot10^6$	$4\cdot10^6$	$8\cdot10^6$	$5\cdot10^5$	10^6	$2\cdot10^6$	$2{,}5\cdot10^6$	$5\cdot10^5$	10^6	$2\cdot10^6$	10^6	10^6
1:10	+	++	+++	++±	+++	++±	+	++	+++	+++	+	++	+	+	+
1:20							+	++	++±	+++	+	++±	++±	+	+
1:40							+	++	++±	++±	+±	++±	+++	±	—
1:80							+	+±	++	++	+±	+±	++	—	—
1:160							+	+	+	+	+	+	+±	—	—
1:320							±	+	+	+	±	+	+	—	—
1:640															
1:1280															

Note: The indicated dilutions of normal rabbit sera do not agglutinate cells from normal mouse organs and mouse tumors.

TABLE 2. The Use of Different Reaction Media in Agglutination of Mouse Tumor Cells

Serum dilution	Immune serum against mouse sarcoma SaI	
	versene buffer 0.005M (pH 7.0)	dextran, human serum
	sarcoma SaI	
1:20	++++	++++
1:40	+++	+++
1:80	++	+++
1:160	++	++
1:320	+	+-
1:640	+	+±
1:1280	±	+
1:2560	—	+
1:5120	—	±

Note: The indicated dilutions of normal rabbit sera do not agglutinate mouse sarcoma cells.

ml flasks. In the morning, the pieces are trypsinized at 18° in a magnetic stirrer 1-3 times for 10-30 min, depending on the consistency of the tumor. In the case of a very solid tumor, the flask with the pieces is incubated for additional 10-30 min at 37° (the time of heating and the trypsinization procedure are determined experimentally for each tumor). Immediately after every trypsinization the cell suspension is diluted no less than 3-5 fold with cold Ringer's solution (occasionally with addition of 1% normal serum to neutralize trypsin activity) and is centrifuged at 900 rpm for 5 min. The sediment is suspended in Ringer's solution and is filtered through two layers of gauze. The obtained cell suspension is washed 4-5 times with Ringer's solution, suspended in the reaction medium (see below) and the viable cells are counted by straining with 0.05% eosin solution [19]. The suspensions prepared by this technique usually consist of single cells, 75-93% of which are viable.

Cells of ascites tumors are suspended in 1% solution of sodium citrate in Ringer's solution, washed free of leucocytes, erythrocytes and ascites fluid using 5-6 cycles of centrifugation, and the sediments are suspended in Ringer's solution without citrate. The sediment after final centrifugation is suspended in the reaction mixture and the number of viable cells is determined. Usually 95-96% of cells remain viable. Cervical, axillary, brochial, and inquinal lymph nodes of mice (in all 10 lymph nodes from a single mouse) devoid of fat and connective tissue, and used to prepare mouse lymphocyte suspensions. The lymphocyte suspension may be prepared by different methods: by careful fragmentation of the lymph nodes with scissors, by careful grinding in a mortar, or by passing through a 40 × 40 mesh metal sieve attached to a special disintegrator. Analogous results are obtained using these methods: 30,000,000 to 75,000,000 lymphocytes are obtained from an adult mouse (depending on the strain), of which 75-80% are viable. Careful grinding of the lymph nodes in a mortar is preferred, inasmuch as this procedure (suggested by O. M. Lezhneva) is the least time consuming. The lymphocytes are suspended in Ringer's solution, allowed to sediment for 3-5 min., the supernatant fluid, containing usually only occasional cells, is removed by suction, washed 4 times in Ringer's solution by centrifugation at 1100 rpm for 5 min and suspended in the reaction medium.

Spleen, liver and kidney cell suspensions may be prepared in the same manner, but these suspensions always contain a significant admixture of erythrocytes. In order to prevent spontaneous agglutination, it is important that the suspensions should contain a minimum number of dead cells.

We used different concentrations of the tumor and normal cells in agglutination reaction (Table 1), The optimum concentration of the tumor cells in the test tube is 1,000,000 per ml and that of the spleen cells is 2,500,000 per ml. Lower concentrations give less definite agglutination and the use of greater concentrations leads to difficulty in ascertaining the reaction.

The relative volumes of immune serum and cell suspension in the test tube are not as significant as is claimed by Kite and co-workers. It is preferrable to mix equal volumes (0.1 ml of each) of every serum dilution and cell suspension. Here, the final concentration of tumor cells must be 2,000,000 per ml and of normal cells 5,000,000 ml.

The optimum reaction mixture for agglutination of normal cells has been proposed by Kite and co-workers to be 0.005 M solution of versene in physiological solution (pH 7.0). For agglutination of the tumor cells, it is better to prepare the versene buffer in Tyrode's solution: this medium results in less pronounced "granules" in the control. The serum dilutions are prepared in the same medium used for suspending cells.

The optimum condition for incubation is not less than 2 days at 4°. There is no need to resort to prior incubation at 37°.

Agglutination is recorded through an agglutinoscope; occasionally it is necessary to use a microscope for recording the final dilution.

In addition to the described procedure, we used that suggested by Gorer and Mikulska [8] for determination of incomplete isohaemagglutinins. Dilution of serum is made in 2% solution of dextran in physiological solution (6% dextran solution in 5% glucose, supplied by "Intradex" is used as the basic preparation); the cells are suspended in normal human serum, inactivated and adsorbed with erythrocytes and mouse organs and diluted 1:2 in physiological saline.

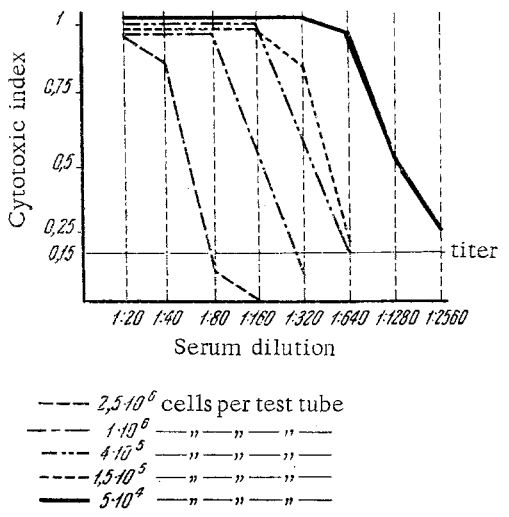

Fig. 1. The dependence of the cytotoxic index upon the number of tumor cells used for the cytotoxic reaction in-vitro. Cytotoxic index $= \frac{a-b}{a}$, where a = the number of viable cells in the control; b = the number of viable cells in the experiment [9].

Other details of reaction procedure were analogous to those described. A mixture of different volumes of solution of the same dextran and cell suspension in normal human serum served as control.

It has been possible to demonstrate that sensitivity of the agglutination reaction increased 4 fold using this method, i.e. by 2 dilutions (Table 2). Introduction of only dextran in the medium (without human serum) did not have this effect.

The cytotoxic reaction in vitro was proposed by Gorer and O'Gorman in 1956 [7] for identification of isoantigens. Subsequently, this reaction has been used by many investigators, introducing individual modifications of the method [8, 9, 16, 18, 20, 22, 23, 25, 26]. Nevertheless, according to the authors who suggested this procedure, it has a limited sensitivity [8].

We investigated a number of parameters of the reaction in our attempts to increase its sensitivity; the number of cells, the dilution of complement, the time and temperature of incubation, and variations in experimental design. The experiments were carried out in a homologous and heterologous system; in the present communication we offer the results obtained using a heterologous system.

Mouse sarcomas SaI, induced and developing either as ascites or solid states in inbred mice of strain A (obtained from the U.S.A. from Professor Kaliss) were used in the experiments, as well as sera from rabbits immunized with this sarcoma. The cell suspension was prepared using the above described procedure. Guinea pig sera showing a minimum non-specific cytotoxic effect on the tumor cells after incubation for $1\frac{1}{2}$ h at 37°, have been used as complement. The complement was preserved at -20°.

The use of different cell concentrations in the reaction indicated that decreasing the number to 5×10^4 cells per test tube significantly increases the sensitivity of the reaction, compared to the routine procedure using 1×10^6 cells per test tube. This leads to an increase in the cytotoxic index (Fig. 1) as well as in the serum titer (Fig. 2). The final serum titer increases 16 fold (by 4 dilutions) using this procedure.

However, it became necessary to alter the procedure used for the reaction, in order to establish the relative number of viable and dead cells in such a small total concentration in the test tube. We tried several possibilities and believe the following procedure to be most suitable.

To 0.05 ml of the respective dilutions of immune serum (prepared in Ringer's solution) are added 0.05 ml of cell suspension in Ringer's solution, containing 1×10^6 cells per ml (of which 80% are viable), i.e., to each test tube are added 5×10^4 cells. A mixture of the same volumes of the cell suspension and 10% normal rabbit serum in Ringer's solution is used as a control. The mixture of cells and serum is incubated for 30-40 min at 18 or 37° (the temperature has no significant influence on the reaction), followed by the addition of 0.1 ml of complement, which is therefore, diluted in half. The mixture with the complement is incubated for 90 min at 37° (incubation for 18 h at 4° results in similar cytotoxic reaction, however, it may be accompanied by non-specific cytotoxicity of the complement). After incubation the test tubes are centrifuged at 900 rpm for 5 min, the supernatant fluid is removed, and 0.06 ml of Ringer's solution containing 50 mg of aqueous eosin and 50 mg of trypan blue per 100 ml is added to the sediment and the ratio of the unstained (live) and stained (dead) cells is determined. The centrifugation is essential, since it allows a small amount of stain to be added to the sedimented cells, which makes it possible to enumerate a small number of cells without lowering the significance of the operation. The cytotoxic index is calculated according to Hellstrom's method [9]. The highest dilution of serum giving the index of 0.15 is considered to be the titer.

The above variation of the procedure for cytotoxic reaction, not reducing the sensitivity of the agglutination reaction, improves upon it in relation to the greater objectivity of establishing the antiserum titer. The relatively

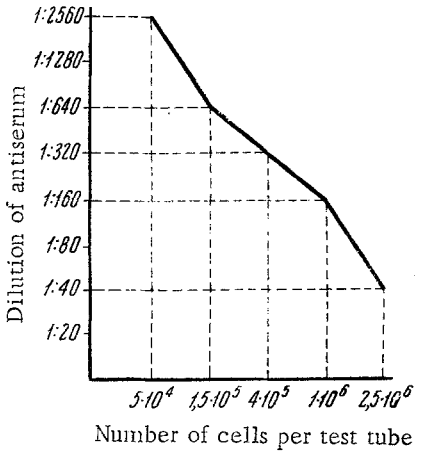

Fig. 2. The dependence of the antibody titer in the cytotoxic reaction in vitro upon the number of tumor cells used in the reaction.

high sensitivity of the above method has been demonstrated by us using a homologous system; it allowed to establish the cytotoxic effect of isoantibodies in relation to the "resistant" sarcoma SaI, which cannot be achieved with the usual method [16, 26].

Upon completion of the present study, there have been published two papers [4, 12] with similar results in relation to the cytotoxic reaction in vitro.

Therefore, the results of the current study are the refinements and development of the optimal conditions for agglutination reaction of the tumor and normal cells and of the cytotoxic reaction in vitro.

Variations of the routine procedures for these reactions are proposed which allow to increase their sensitivity several fold.

SUMMARY

Optimal conditions for agglutination reaction of the tumor and normal cells of mice and cytotoxic reaction in vitro were ascertained and developed. Equal volumes (0.1 ml) of antiserum and cellular suspension, containing 2 million per ml of tumor cells or 5 million per ml of lymphocytes (not less than 80% of the cells should be living) were used for the agglutination reaction. Incubation —2 days at 4°C. Reaction medium is 0.005 M solution of versene in physiological saline—pH-7 (for normal cells) and in Tyrode solution (for tumor cells). The use as a reaction medium of the system "dextran-human serum" increases 4-fold the reaction sensitivity.

With the cytotoxic reaction in vitro, the reduction of the cell dose down to $2.5-5\times10^4$ per test tube and the subsequent thereto modification of the method for staging the reaction permits it to increase 6-fold its sensitivity as compared to the accepted experimental method.

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