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DETERMINATION OF HYPERSENSITIVITY TO ANTIGENS BY COUNTING CELLS MIGRATING FROM CAPILLARY TUBES

D. K. Novikov, G. P. Adamenko, and V. I. Novikova

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Migration of leukocytes and macrophages from capillary tubes placed in a well containing medium was measured by counting the number of cells coming out into the medium. The method is highly sensitive and enables a small number of cells to be used with a large number (up to 600 or more) of capillary tubes. Sodium azide, phytohemagglutinin, and incubation at 4°C inhibited migration of the cells from the capillary tubes. Dead cells did not migrate from the tubes into the medium. Tuberculin and Bacillus Calmette-Guerin (BCG) vaccine inhibited migration of macrophages of guinea pigs immunized with BCG but not with staphylococcal allergen, and vice versa.

KEY WORDS: hypersensitivity of delayed type; migration of cells from capillary tubes.

Determination of migration of leukocytes and macrophages has been extensively applied in the study of reactions of hypersensitivity of delayed type (HDT). In this state, sensitized lymphocytes cultivated *in vitro* with the specific antigen liberate substances which inhibit or, less frequently, stimulate the migration (mobility) of leukocytes and macrophages [8, 12, 13]. Existing methods of determination of this state and evaluation of the inhibition of cell migration are based on measurement with a planimeter or by projecting on paper the zones of cells migrating onto the surface of a slide from horizontally placed capillary tubes in special chambers [2, 7, 14, 18, 19], from fragments of the spleen and clots of plasma [3, 10,

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20], or from wells in a gel [11]. Migration of cells is also determined by measuring the increase in length of their residue in capillary tubes [1, 15]. These methods are laborious and inefficient, for they require special chambers and special arrangements for mounting the capillary tubes in them and for measuring the zones of migration of the cells; the latter is largely subjective and does not take into account the density of distribution (number) of migrating cells, and so on. These factors combine to reduce the sensitivity and reproducibility of the reaction, and this has led some workers [15, 16] to regard it as unsuitable for the detection of HDT.

The object of this investigation was to reduce the laboriousness of the method of determining cell migration, to make it simpler and more sensitive, to reduce the number of cells required for the reaction, and to automatize the reading of the results.

The inhibition of leukocyte migration test (ILMT) as suggested by the writers can be used to study migration not only of blood leukocytes, but also of cells from the peritoneal exudate of animals and also cells of other origin. It consists of several stages which, except the reading of the results, are carried out under strictly aseptic conditions using sterile material.

EXPERIMENTAL METHOD

Preparation of the Cell Suspensions. Cells of the peritoneal exudate of animals were obtained 3-4 days after intraperitoneal injection of 10% gelatin solution or sterile mineral oil in a dose of 2 ml into mice or 10-20 ml into guinea pigs. The peritoneal cavity of the animals was irrigated with 10-50 ml of Hanks's solution. The cells were washed with this solution by centrifugation at 1000 rpm for 5 min each time.

To obtain human blood leukocytes the following procedures were used. Blood (1-10 ml) was taken from a vein into tubes containing heparin (20 units/ml). The top layer of plasma, rich in leukocytes, was aspirated after allowing the blood to stand for 1-3 h and it was centrifuged at 1000 rpm for 5 min. The plasma was drawn off and again centrifuged at 2000 rpm for 5 min, and the residue was treated with 9 volumes of 0.84% ammonium chloride, freshly made up in Tris-HCl buffer [9] or distilled water, and the cells were resuspended. Under these hypotonic conditions any red cells present were hemolyzed in 2-3 min. The suspension was centrifuged at 500 rpm for 5 min, the supernatant was poured off, and the cells were washed twice with medium No. 199. A suspension was made from the residue of leukocytes with a concentration of 5-10 million cells/ml in medium No. 199 containing 20% autologous or homologous plasma or bovine serum (depending on the object of the experiment), with the addition of 100 units/ml each of penicillin and streptomycin.

Filling the Capillary Tubes. Glass or plastic capillary tubes can be used. We usually used glass capillary tubes, not silicone-treated, 0.8 cm long and with an internal diameter of 1.2 mm. One end of these capillary tubes was first sealed. The experimental and control cell suspensions were divided into two or more equal parts (depending on the number of antigens to be tested for). To one part of the cell suspension was added the solvent of the antigen, and to the others the various substances or antigens to be tested for in concentrations nontoxic for cells. At least five capillary tubes were filled with each type of suspension. For this purpose, through the open end of the capillary tube about 0.02 ml of the cell suspension was introduced from a syringe through a subcutaneous injection needle so that the meniscus of the fluid at the end of the capillary tube was concave. The capillary tubes were lowered with their open end on the floor of a series of wells measuring 0.6 × 1 cm made in transparent plastic plates measuring 10 × 20 cm. Into each well 0.05 ml of the same medium as was used to prepare the cell suspensions was poured initially. The plates were covered with glass plates measuring 9 × 19 cm and their edges were sealed with paraffin wax. The plates were incubated at 37°C for 6-24 h.

Reading the Results. After incubation the capillary tubes were carefully removed from the wells with forceps and cells migrating from them and settling on the floor of the well were resuspended with a pipet. The concentration of cells in the medium was then determined by the usual method in blood cell counting (Goryaev's chambers) or by means of automatic blood cell counters. The last method of counting is particularly effective if the number of capillary tubes used is large (400-600). After the cells were counted the mean number was calculated (migration index) for a group of five capillary tubes and the significance of differences between the means was determined by Student's method. If the substance or antigen added to the test cell suspension inhibited or stimulated migration, the mean number of cells

TABLE 1. Effect of Treatment with Ethanol and of Temperature and Duration of Cultivation of Leukocytes on Their Migration from Capillary Tubes (mean results of four experiments with confidence limits at $P < 0.05$)

Treatment of leukocytes and conditions of cultivation	Cell concentration (in millions/ml in wells)	Inhibition of leukocyte migration, %
Untreated		
37°, 18 h	2±0,08	—
4°, 18 h	0,74±0,06	63
37°:		
6 h	0,76±0,03	62*
12 h	1,5±0,04	25*
24 h	2,3±0,12	15*, †
30 h	2,1±0,14	5 †

*Percentage of inhibition of migration calculated relative to leukocytes cultivated for 18 h under the same conditions.
†Stimulation of leukocyte migration compared with the control.

TABLE 2. Inhibition of Migration of Peritoneal Exudate Cells from Guinea Pigs Immunized with BCG Vaccine and Staphylococcal Allergen under the Influence of Those Antigens

Immunization with	Index of inhibition of macrophage migration (in %)		
	BCG vaccine	old tuberculin	staphylococcal allergen
BCG vaccine	40	30	4*
" "	35	32	6*, †
" "	44	40	2*
" "	50	45	0
" "	24	27	1
Staphylococcal allergen	10*, †	2*	26
" "	5*	0	31
" "	2*	3*, †	39

*Differences not significant: $P > 0.05$–0.1; in other cases $P < 0.02$–0.01.
†Stimulation of migration of macrophages.

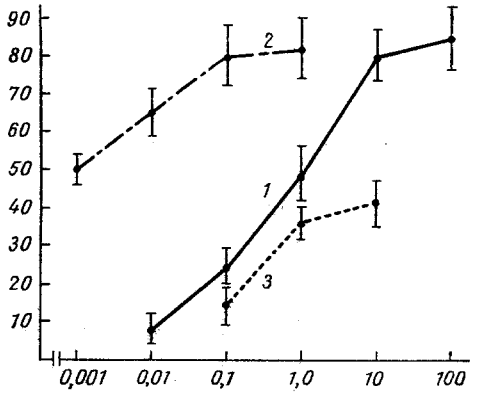

Fig. 1. Effect of phytohemagglutinin (1), sodium azide (2), and puromycin (3) on migration of leukocytes from capillary tubes (mean results of four experiments). Ordinate, inhibition of migration of leukocytes (in %); abscissa, concentration of substances (in μg/ml).

in the wells with medium in this group of capillary tubes would be significantly smaller or, conversely, larger than when the same suspension was used without the antigen. Meanwhile it must not have affected the suspension of control (unsensitized) cells. The effect of the antigen was determined by the equation

$$\text{Index of inhibition of leukocyte migration (in \%)} = 100 - \frac{\text{Index of migration with antigen}}{\text{Index of migration without antigen}} \times 100.$$

Effect of Certain Conditions and Agents on Leukocyte Migration. The capillary tubes were placed in the wells in a sloping position (70-80°) and, for that reason, most of the cells settled on their wall. Only a few cells, at the open end of the capillary tubes, could fall "passively" into the medium contained in the wells. For cells to migrate actively from the capillary tube into the medium, they had to cover a certain distance to the end of the capillary tube. The migration activity of the cells was determined from their ability to travel along this path in a certain time, estimated from their migration from the capillary tube by

counting the number of cells appearing in the medium in the well. With this approach there was no need to measure the migration zones of the cells or to use special chambers.

The following facts indicate that migration of cells from the capillary tubes is an active process requiring normal cell metabolism. The leukocytes of human blood and macrophages of guinea pig peritoneal exudate, when treated with 96% ethanol (i.e., dead), and then washed with medium No. 199 did not migrate from the capillary tubes into the medium. Their number in the medium in the well was less than 10% of the number of living cells of the same suspension contained in capillary tubes and incubated similarly. At 4°C migration of leukocytes from the capillary tubes was sharply inhibited compared with incubation of the same cell suspension in the capillary tubes at 37°C (Table 1). Puromycin inhibited migration of blood leukocytes (Fig. 1). Sodium azide, which inhibits cell metabolism and respiration, in a concentration of 0.001-1 μg/ml, sharply inhibited migration of cells from the capillary tubes (Fig. 1). Phytohemagglutinin P (1-100 μg/ml) also inhibited migration of blood leukocytes and of cells from fragments of the spleen [4]. The optimal conditions for migration were incubation at 37°C for 18-24 h and the presence of 10-20% blood serum in the incubation medium. With a decrease in the incubation time fewer cells migrated from capillary tubes into the medium in the well. The relative percentage of granulocytes and lymphocytes migrating from the capillary tubes was not substantially altered by comparison with the original suspension.

The method was used to detect bacterial allergy. Five guinea pigs were immunized with 0.1 mg BCG vaccine in the footpad. Two weeks later peritoneal exudate cells were obtained from these and three other guinea pigs which had been immunized with staphylococcal allergen (with four skin doses). One part of the suspension of guinea pig cells was treated with old tuberculin (final dilution 1:500), another part with BCG vaccine (final concentration 0.001 mg/ml), and the third part with staphylococcal allergen (0.05 skin dose/ml). Capillary tubes were filled with the suspensions and the test carried out as described above. Both tuberculin and BCG vaccine inhibited migration of leukocytes from guinea pigs immunized with BCG vaccine but not with staphylococcal allergen, and vice versa (Table 2).

By the use of this method the writers previously demonstrated tumor-specific sensitization of the leukocytes of cancer patients [5] and also revealed sensitization of healthy persons and of patients with tuberculosis to mycobacterial antigens [6].

The suggested method can be used to determine the migration activity of cells and also to detect allergy in cases when existing methods of inhibition of migration of macrophages and leukocytes such as may be used to study HDT are employed (bacterial and drug allergy, autoallergy, parasitic diseases, transplantation immunity, immunodiagnosis of tumors, etc.).

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INTERACTION BETWEEN ANTI-K^b AND ANTI-D^d EFFECTOR LYMPHOCYTES AND TARGET CELLS OF MICE OF MUTANT HAPLOTYPES

A. V. Andreev, G. I. Drizlikh,
B. D. Brondz, and I. K. Egorov

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The cytotoxic action of anti-K^b (C57BL/6) and anti-D^d (B10.D2) immune lymphocytes on target cells of K^{ba} (Hz1) and K^{bd} (M505) mutants and also on D^{da} (M504) target cells was greatly reduced compared with their cytotoxic action on target cells of the original C57BL/6 and B10.D2 strains, respectively. The decrease in cytotoxic action was more marked on H(z1) and M504 than on M505. By adsorption on a monolayer of mutant target cells, monospecific anti-K^b and anti-D^d lymphocytes can be subdivided into subpopulations, one of which reacts only with target cells of the original strain, the other with target cells of both the original and mutant strains.

KEY WORDS: H-2 histocompatibility complex; mutations of the H-2 complex; alloantigenic specificities; receptors of effector lymphocytes.

The H-2K and H-2D loci of the basic histocompatibility complex of mice encode both special and general serologically determinable specificities (SDS) [15] and also determinants identifiable by the receptors of cytotoxic T lymphocytes (CTL), causing rejection of grafts *in vivo* and destruction of target cells *in vitro* [10]. It is not yet clear whether the SDS and the structures identified by the CTL are the same or different determinants. The writers showed previously that CTL receptors do not recognize the general but react either with the special SDS or with "CTL determinants" closely connected with them [6]. To study this problem further, it was decided to use mutants of the K^b allele, namely H(z1) (K^{ba}) and M505 (K^{bd}), derived from C57BL/6, and a mutant of the D^d allele, namely M504 (D^{da}), derived from B10.D2. In both cases reciprocal immunization between the original and mutant strains leads to graft rejection and induction of CTL [4, 9, 11, 12], despite the fact that special SDS of normal K^b (H = 2.33) and D^d (H = 2.4) haplotypes were preserved in the mutants, although evidently in somewhat reduced amounts [1, 17] or in a slightly modified form [8].

The object of this investigation was to study whether CTL directed against products of the normal K^b or D^d alleles can distinguish target cells of the original and mutant strains.

EXPERIMENTAL METHOD

Mice of strain C57BL/6 (K^bD^b) (abbreviation B6) and of the recombinant strain R101 (K^dD^b) and also of mutant strains M505 ($K^{bd}D^b$), H(z1) ($K^{ba}D^b$), and M504 (K^dD^{da}) were maintained in the Laboratory of Genetics of Tissue Compatibility, Institute of General Genetics. Mice of strain B10.D2(K^dD^d), abbreviation D2, and C57BL/10 (K^bD^b), abbreviation B10, were obtained from the nursery of the N. F. Gamaleya Institute of Epidemiology and Microbiology.

The ascites form of sarcoma MKh11 and the solid form of sarcoma MKh26, induced by methylcholanthrene in B10 and D2 mice, respectively, were maintained by regular passage.

Laboratory of Immunochemistry and Diagnosis of Tumors and Laboratory of Chemistry and Synthesis of Antibodies, N. F. Gamaleya Institute of Epidemiology and Microbiology. Laboratory of Genetics of Tissue Compatibility, Institute of General Genetics, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR M. A. Baroyan.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 81, No. 6, pp. 710-713, June, 1976. Original article submitted August 20, 1975.