EFFECT OF CONDITIONS OF CULTURE AND MITOGENIC STIMULATION ON HLA ANTIGEN EXPRESSION IN LYMPHOCYTES

A. Yu. Volgin

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One approach to the study of the biological role of the principal human histocompatibility complex is to investigate expression of HLA antigens on cells cultured *in vitro*. By now HLA antigens have been found on the membrane of virtually all nucleated cells, but the degree of expression of gene products of the chief histocompatibility complex and their qualitative composition vary considerably in different tissues [2-4].

Histocompatibility antigens can be typed by various methods. The most widely used method is the complement-dependent lymphocytotoxic test [2]. Most investigations to study population differences in the distribution of HLA antigens are carried out by this method and associations of HLA antigens with diseases have been established. However, because of technical difficulties the lymphocytotoxic test has been used much less frequently to study cells cultured *in vitro*. For example, the typing of lymphoblastoid cells by this method is complicated because of the nonspecific cytotoxicity of rabbit complement toward them [6, 8]. The expression of HLA antigens, if undertaken by various methods, is also made more difficult by the necessity of comparing and interpreting results obtained when typing cells cultured *in vitro* and also obtained by the study of different population groups of healthy subjects or patients.

In the investigation described below the lymphocytotoxic test was used to study expression of HLA antigens on lymphocytes cultured *in vitro* and on lymphocytes treated with mitogens and cytostatics.

EXPERIMENTAL METHOD

Lymphocytes were isolated from heparinized blood (100 i.u. heparin/ml) from healthy donors by means of a Verografin – Ficoll gradient (D = 1.077). The cells were cultured in plastic trays from the Leningrad Medical Polymers Factory. The cell concentration in the suspension was $1.5 \cdot 10^6$/ml. The culture medium was RPMI 1640 medium (from Flow Laboratories, England) with the addition of antibiotics (100 i.u./ml of penicillin and of streptomycin), 10% embryonic calf serum (from Serva, West Germany), and 10% human group AB blood serum (pool from four donors). The sera were inactivated by heating to 56°C for 40 min. HEPES was used as the buffer. The lymphocytes were cultured for 72 h. ^{3}H-Thymidine was added to the culture in a dose of 1μ Ci/ml 20 h before the end of cultivation. The presence of HLA antigens on the lymphocytes was then determined and some of the cultures were transferred to Synpor membrane filters and washed with 10 volumes of Hanks' solution, 7% TCA, and 96° ethanol. After drying, the filters were placed in flasks containing ZhS-1 scintillation fluid for counting. Activity of the incorporated label was counted on an Intertechnique SL-30 scintillation counter for 1 min.

Phytohemagglutinin (PHA) from Difco, USA (10 μg/ml), concanavalin A (Con A), from Pharmacia, Sweden (15μg gml), and B lymphocyte mitogen (PWM), from Sigma, USA (30 μg/ml) were used as mitogens. The Soviet preparation of cyclophosphamide (cyclophosphan) and its active metabolites were used as the cytostatic. The source of activated cyclophosphan was serum from (CB × C57BL/6)F_1 mice, into which cyclophosphamide was injected intraperitoneally in a dose of 300 mg/kg body weight 30 min before sacrifice. The alkylating activity of the serum was determined by the NBP test [5], using the modification in [1], and the serum was kept at –70°C until use. In the case of treatment with activated cyclophosphamide, the lymphocytes were incubated before culture with serum containing in a final dilution 0.6 unit of alkylating activity/ml for 1 h at 37°C. After incubation the lymphocytes were washed with medium and their viability determined.

To identify histocompatibility antigens the lymphocytotoxic test suggested by Terasaki [12] was used, with partial modification as in [13]. HLA antigens were identified by two sets of typing sera, corresponding to International Serum Standards, some obtained from the Institute of Medical Genetics, Academy of Medical Sciences of the USSR, others from

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TABLE 1. Effect of Conditions of Culture and Mitogenic Stimulation of Lymphocytes on Detectability of HLA Antigens

Lymphocytes	Mean number of antisera reacting to one antigen		Mean number of cross-reacting antisera to one antigen	
	anti-HLA-A	anti-HLA-B	anti-HLA-A	anti-HLA-B
Before culture	2,7	2,7	0,5	2,2
After culture	4,4	4,6	1,2	3,2
Stimulated by PHA	4,8	5,2	2,7	3,5
Stimulated by Con A	4,7	6,0	4,0	3,0
Stimulated by PWM	3,0	6,3	3,0	4,0

TABLE 2. Effect of Treatment with Activated Cyclophosphamide on Stimulation of Lymphocytes by Mitogens*

Lymphocytes	Without stimulation	Stimulated by		
		PHA	Con A	PWM
Untreated	1,0	8,3	5,3	4,6
Treated with cyclophosphamide	1,1	2,1	1,3	1,2

*Values of index of stimulation (ratio of number of cpm in stimulated culture to number of cpm in unstimulated culture) given in this table.

TABLE 3. Effect of Treatment with Activated Cyclophosphamide on Detectability of HLA Antigens

Lymphocytes	Mean number of antisera reacting to one antigen		Mean number of cross-reacting antisera to one antigen	
	anti-HLA-A	anti-HLA-B	anti-HLA-A	anti-HLA-B
Before culture	2,7	2,7	0,5	2,2
After culture	4,4	4,6	1,2	3,2
After treatment with cyclophosphamide and culture	1,5	2,5	0,3	2,0
After treatment with cyclophosphamide and culture with PHA	1,0	2,0	1,0	1,,0

research groups headed by Professor Dausset (France) and Professor Kissmeyer-Nielsen (Denmark). With both sets of sera it was possible to identify 33-35 antigens of the HLA-A and HLA-B series.

The results were subjected to statistical analysis, using the Wilcoxon–Mann–Whitney U criterion.

EXPERIMENTAL RESULTS

The experiments on typing HLA antigens showed that the lymphocytotoxic test can be used successfully to determine histocompatibility antigens on lymphocytes cultured *in vitro*. As a result of culture of the lymphocytes more of the HLA antisera gave a clear reaction with the cells, but the specificity of the direct and cross reactions was unchanged (Table 1).

It will be clear from Table 1 that after culture of the lymphocytes the number of reacting antisera against HLA antigens of both series was increased. The same effect also was found with regard to cross-reacting antisera. For instance, the mean number of antisera reacting with HLA-A antigens was 2.7 before culture and 4.4 after culture. The corresponding values for antigens of the HLA-B series were 2.7 and 4.6. Addition of mitogens to the culture medium increased the number of reacting antisera even more; moreover, as Table 1 shows, this effect was most marked during typing of antigens of the HLA-B series. The mean number of antisera reacting with the cells was 5.2 for cells treated with PHA, 6.0 with Con A, and 6.3 with PWM.

Treatment of the lymphocytes with activated cyclophosphamide did not kill the cells but considerably inhibited cell proliferation due to mitogens (Table 2). Moreover, this treatment completely abolished the effect of an increase in the number of reacting HLA antisera culture of the lymphocytes. Stimulation by mitogens of cyclophosphamide-treated lymphocytes did not lead to any increase in the number of positive reactions (Table 3).

There may be various causes of the increase in the number of reacting antisera.

One possible mechanism may be that under the influence of factors in the culture medium or of mitogens, a conformational redistribution of HLA antigens takes place on the lymphocyte membrane. As a result of this change the

"common" sites of HLA antigens, whose presence has been demonstrated in a number of investigations [10, 11], become more accessible to the action of antisera. The greater accessibility of the "common" site of individual antigens may also explain the increase in reaction of cross-reacting antisera. The existence of a mechanism bringing to light previously functionally "silent" determinants is confirmed by the results of investigations [7] which showed expression of DR antigens on T cells after heating for 1 h at 45°C. Before heating, the DR antigens could not be found on the cells by means of antisera. The possibility of masking of HLA-A and HLA-B antigens has been noted by other workers [2]. According to their findings, the ability of platelets to react with antisera increases with an increase in the length of keeping of the material and reaches a maximum by the 4th-8th day of keeping.

Another possible explanation of this phenomenon may be the suggestion that lymphocytes become more sensitive to the cytotoxic action of the antibody – complement complex. It has been shown [6, 8], for instance, that lymphoblastoid cells of continuous cell lines become sensitive to and die from the nonspecific cytotoxic action of rabbit complement. In this way blast transformation of lymphocytes causes the appearance of "weak" reactions, which usually do not lead to death of peripheral blood lymphocytes. However, it is still not quite clear why the effect of an increase in the number of reactions should be given by unstimulated lymphocytes and why treatment with cyclophosphamide should lead to the abolition of this phenomenon.

The third possible mechanism may be that factors stimulating synthesis of HLA antigens act on the lymphocytes during culture. Treatment with mitogens stimulates this process even more. The fact that the number of HLA antigens can be changed has been shown in experiments on fibroblasts [9]. These showed that the number of HLA antigens, expressed by the same cell, depends on the phase of the cell cycle. The smallest number of HLA antigens is expressed in the G_0 phase, the largest in the S phase. In the present investigation most lymphocytes stimulated by mitogens were in the S phase at the moment of typing.

The active character of the mechanism lying at the basis of the increase in the number of reacting sera is confirmed by results of experiments with activated cyclophosphamide, in which this effect was completely suppressed and the mitogenic reaction of the lymphocytes was considerably inhibited.

The results indicate that the sensitivity of human lymphocytes to the specific action of HLA antisera depends not only on the properties of the sera, but also on the functional state of the lymphocytes themselves. A change in this state evidently affects expression of HLA antigens and (or) their accessibility to the action of HLA antibodies. Culture of lymphocytes with subsequent typing can be recommended in cases when precise determination of phenotype is needed but the number of positive reactions is insufficient for a final decision. This may arise, for example, during determination of HLA antigens in family studies, for the establishment of paternity, and so on.

In the writer's view, the further study of the mechanism of potentiation of the actions of lymphocytes with antisera will help toward the understanding of the mechanisms of function of antigens of the principal histocompatibility complex.

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