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Reevaluation of Migration Inhibition Indices in the Immunological Study of Mice Fibroblast Transformed Spontaneously in Vitro*

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Summary

The immunological reactivities of St/a mice fibroblast transformed in vitro (STL-1), Ehrlich ascites tumor cell, St/a ascites tumor cells (STABAL), non-transformed but transplantably conversed St/a mice fibroblast (STL-22) and C3HL-1 cell were examined by the migration inhibition test. In this test performed by the microcapillary tube technique, spleen cells and mixtures of thymus and lymph node cells were used as migration cells.

From this study, it was concluded that the usual calculation of migration indices is unlikely to reveal a distinctive tendency. When cases in which the migration inhibition indices (MII) of non-sensitized lymphoid cells are lower than those of sensitized lymphoid cells were excluded from the evaluation of the migration inhibition test, the results of the migration inhibition test correlated well with the results of two other immunological examinations, immunofluorescent and active protection test. Unpurified spleen cells is not suitable for the capillary migration inhibition test.

It was also noted that anti-STL-1 sera did not affect the antigenic target cells, but acted directly on the lymphoid effector cells, and showed antigenic inhibitions in the migration inhibition test.

Introduction

Migration inhibition tests with peritoneal macrophages and/or leukocytes have frequently

been used in immulogical studies in human and animals. The evaluation in these tests is usually based on the calculation of Migration Index (MI) or percent Migration Inhibition.

In most cases the normal migration zone is taken as 95% of the area in which non-sensitized control migration cells without antigens accumulate. Estimation of this zone may vary depending on the technical accuracy of the investigators. ALVORD[2] took the normal range of MI as 0.90-1.10, but usually it is taken as 0.80-1.20 in leukocyte migration, and 0.70-1.30 in peritoneal macrophage migration inhition tests (SøBORG[27]).

Cases showing migration indices out of this normal range are considered positive. Most positive cases have shown migration inhibition, but a few cases have shown migration enhancement. Specific enhancement of migration is frequently observed when the concentration of antigens is rather low (WEISBART[30]).

The results of experiments are usually shown by the ratio of the number of positive cases to the total number of cases (BODDIE[6], LACOUR[17]). Of course, non-sensitized migration cells should be included as the control in each test. But sometimes these tests lack sensitivity or produce results which are difficult to interpret (ARMISTEAD[3], ROSENBURG[25], REMOLD[24]).

In this paper, lymphoid cells sensitized by STL-1 cells, which had been proved to have a specific immune response with STL-1 cells themselves and also to shown common reactivities with Ehrich, STABAL and C3HL-1, but not with STL-22 cells in the active protection test in vivo, were used and the problems involved in the migration inhibition tests were solved by the exclusion of non-specific migration inhibitions of non-sensitized lymphoid cells.

Materials and Methods

Preparation of lymphoid cells and antigen : St/a Fib. mice were obtained from our colony maintained under 'minimal disease' conditions. Usually two to three month old mice of both sexes were immunized with twice subcutaneous injections of 5×10^6 STL-1 cells on bilateral flanks given one week apart.

Sensitized lymphoid cells were collected from thymus and lymph nodes in the inguinal, axillar, parathymic and submandibular region 7 to 10 days after the last immunization. Spleen cells were not used later because of their inconstant migration (see Table II). These lymphoid tissues were finely cut with scissors, and single cell suspensions were made by homogenizing tissues in a glass homogenizer with a loose fitting pestle, and filtering them through a monolayer nylon mesh of 80 μ pore size. After washing three times with phosphate buffer solution (PBS), the cells were finally washed with migration medium, which will be described later.

The control lymphoid cells were obtained from siblings of the same sex.

STL-1 cells were originally derived from the lung of normal adult mice of inbred St/a Fib. strain. They apparently underwent spontaneous malignant conversion, as evidenced by their round shape and their tumorigenicity in newborn St/a Fib. mice. But they failed

to grow in adult non-treated recipients even at an inoculation dose of 10^8 cells.

In addition to these properties, STL-1 cells are also antigenic, as was previously reported by one of us (KIELER[15]), after hundreds in vitro subcultures over more than 4 years. Ehrich and STABAL cells were kept by in vivo passage.

C3HL-1 cells were derived from lung of normal adult C3H mice and kept by alternative passage in vivo and in vitro.

STL-22 cells were derived from the same origin as STL-1, and underwent malignant conversion to show rapid growth and tumorigenicity to non-treated adult syngeneic mice without morphological change.

Migration Inhibition Test (MIT) : Detailed technical procedures have been reported elsewhere (ODA[21]). Briefly, medium 199, containing 15% heat inactivated horse serum, 100 iμ/ml penicillin, and 100μg/ml streptomycin, was used as migration medium. However, a recent study has revealed thaf 10% horse serum plus 4% mouse serum is optimal for mouse cells, although 15% horse serum is optimal for rat and human cells.

Lymphoid cells were resuspended in migration medium up to 7 times of the original cell pack volume. These cell suspensions were drawn with a vacuum pump into the Drummond microcapillary tubes (20 ul), one end of which was presealed by heating. After centrifugation, the tubes were cut at the fluid-cell interface and the part containing lymphoid cells were fixed with silicon grease in the migration wells of Sterilin No. 308 migration plates (Richmond, U.K.).

By our procedure, 6-8 capillary tubes can be prepared from the lymphoid tissue of a mouse.

Intact antigenic cells were harvested from in vitro or in vivo milieu and washed twice with PBS. An antigenic cell concentration of 10,000 cells/ml was used. After 22 to 24 hours incubation in a CO_2 incubator, migration areas were projected, drawn and weighed.

Each group consisted of 6 capillary tubes. And migration Indices (MI) were calculated as follows :

$$\text{MIGRATION INDEX (MI)} = \frac{\text{each individual migration area}}{\text{mean migration area of 6 samples}}$$

It is known from our preliminary examinations of 434 samples that 95% of control mouse lymphoid cells with or without antigen showed a value between 0.70--1.30. Therefore, the samples which showed MI either higher than 1.30 or lower than 0.70 were excluded from the calculation of Migration Inhibition Indices.

$$\text{MIGRATION INHIBITION INDEX (MII)} = \frac{\text{migration area with antigen}}{\text{mean migration area without antigen}}$$

Cases in which MII was significantly different from that in non-sensitized control rats in two tailed Student's t-test (p$\leq$0.05) were considered positive.

Results

Cross-reactivities of each type of sensitized lymphoid cells were examined and

summarized in Table I. Positive cases were determined by the criterion mentioned above. The reactions of anti-Ehrlich cells appeared weak. But even with the STL-1 system, which is known to be strongly antigenic from immunofluorescence and in vivo active protection test (KIELER[15]), only 69.4% of samples showed positive reactions. The chi square test is not suitable for comparing the results because of the small number experiments. It is difficult to compare these results with that of in vivo active protection test. It is also difficult to see any distinctive tendency from Table I.

Table. I. Migration Inhibition Tests of Sensitized Mice Thymus and Lymph Node Cells

migration cells \ antigen	STL-1	Ehrlich	STABAL	STL-22
anti-STL-1	34/49*(69.4%)	4/6 (66.7%)	1/5 (20%)	4/6 (66.7%)
anti-Ehrlich	1/7 (14.3%)	1/3 (33.3%)		
anti-STABAL	4/6 (66.7%)		3/4 (75%)	
anti-STL-22	4/4 (100%)			4/4 (100%)
anti-C3HL-1	1/1 (100%)			

* number of positive cases / number of total cases (percentage)

Accordingly, the mean MII values of each experiment were classified into two groups; the first in which the MII value of the sensitized lymphoid cells was lower than the control, and the second in which the MII value of the sensitized lymphoid cells was higher than the control.

Table II shows the mean MII value of each group recalculated from the MII of each experiment, and the reaction of anti-STL-1 lymphoid cells to STL-1 cells. The number of positive cases and percentage of total cases are shown in the parenthesises. If this migration inhibition test system works well, the control MII value should be around 1.00.

Table. II. MII Values of Sensitized Mice to STL-1 Cells

antigen	STL-1 cell			
	thymus plus lymph node		spleen	
migration cells	anti-STL-1	non-sens. control	anti-STL-1	non-sens. control
I*	0.76±0.18 t=3.6776 p ; 0.0005-- (28/39 ; 71.8%)	0.93±0.22 n=76	0.97±0.09 t=1.3332 p ; 0.15--0.10 (4/8 ; 50%)	1.03±0.09 n=14
II**	0.90±0.18 t=1.7112 p ; 0.10--0.05 (6/10 ; 60%)	0.78±0.13 n=18	0.94±0.14 t=1.3866 p ; 0.10--0.05 (3/7 ; 42.9%)	0.84±0.13 n=12

I* ; MII value of the sensitized lymphoid cells was lower than the control.
II** ; MII value of the sensitized lymphoid cells was higher than the control.
(number of positive cases out of total cases by usual calculation are shown in the parenthesis)

From the results shown in Table II, the following conclusions can be drawn. Firstly, migration inhibition of thymus plus lymph node cells is sufficiently specific, despite the fact that only 71.8% of cases showed positive results by the usual calculations. Secondly, when the MII of non-sensitized control lymphoid cells is lower than MII of sensitized cells, it is not due to the migration enhancement of the sensitized lymphoid cells, but rather to the non-specific inhibition of non-sensitized lymphoid cells.

When the MIIs of sensitized lymphoid cells which were higher than both 1.00 and MIIs of control cells (called real enhanced group) were selected, these enhancements were significantly different from the MII of control cells (Table III). However these enhancements are not specific for the sensitized group, but could be non-specific inhibition of the control.

In spleen cell migration, the result of inhibition was negative (Table II). Moreover the mean MII value of sensitized lymphoid cells was nearly 1.00, though 4 of 8 cases were positive in the usual calculation. In the enhancement of spleen cell migration, the same result was obtained as in the migration of thymus plus lymph node cells. From these results of the STL-1 system, our new classification and calculation of MII was considered to be generally applicable.

Using the same classification, the reaction of anti-STL-1 lymphoid cells to various cell lines was tested. The results are shown in Table IV. Anti-STL-1 lymphoid cells reacted specifically with STL-1 cells. 66.7% of Ehrlich cells, and 75% of STL-22 cells showed positive results by usual calculation, but they were not positive calculated by our new

Table. III. MII of Real-Enhanced Group in Thymus and Lymph Node Cells

antigen	STL-cell	
migration cells	thymus plus lymph node	
	anti-STL-1	non-sens. control
	1.04±0.05	0.88±0.04
	t=5.0619	n=6
	p ; 0.0025--0.0005	
	(2/4 ; 50%)	

Table. IV. MII of Anti-STL-1 Lymphoid Cells to Various Cell Lines

antigen	STL-1 cells		Ehrlich cell		STABAL cell		STL-22 cell	
migr. cells	anti-STL-1	non-sens. control	anti-STL-1	non-sens. control	anti-STL-1	non-sens. control	snti-STL-1	non-sens. control
I *	0.74±0.10	0.90±0.11	0.84±0.17	0.96±0.17	0.57	0.84	0.58±0.11	0.72±0.17
	t=3.2385	n=16	t=1.2224	n=10			t=1.3835	n=6
	p ; 0.0025--0.0005		p ; 0.15--0.10				p ; 0.15--0.10	
	(9/9 ; 100%)		(4/6 ; 66.7%)		(1/1 ; 100%)		(3/4 ; 75%)	
II**	0.91±0.24	0.79±0.19			1.00±0.07	0.95±0.06	0.74±0.28	0.54±0.04
	t=0.6793	n—4			t=1.0911	n—6	t=1.0000	n—2
	p ; 0.30--0.25				p ; 0.20--0.15		p ; 0.25--0.20	
	(3/3 ; 100%)				(0 4 ; 0 %)		(1 2 ; 50%)	

I *
II**} same as Table II

method. These results correlate well with the previous results of the immunofluorescent test (KIELER[15]).

For the cross-control, the reactions of STL-1 cells to anti-STABAL, anti-Ehrlich and anti-STL-22 lymphoid cells were tested (Table V). In this experiment, both anti-STABAL and anti-STL-22 lymphoid cells reacted with STL-1 cells. The migration inhibition of anti-Ehrlich was statistically borderline. These results showed fair correlation with the in vivo active protection test, although there was no strict correlation between results shown in Table IV and V. This might be due to the differences in expression of tumor antigens in vitro and vivo. In the future, the cross-control of the vivo active protection test should be performed to confirm the discrepancy.

Table. V. MII of Various Sensitized Lymphoid Cells to STL-1 Cell

antigen	STL-1 cell		STL-1 cell		STL-1 cell	
migr. cells	anti-Ehrlich	non-sens. control	anti-STABAL	non-sens. control	anti-STL-22	non-sens. control
I *	0.80 ± 0.08	0.86 ± 0.05	0.69 ± 0.06	0.88 ± 0.01	0.77 ± 0.03	$0.91=0.02$
	$t=1.4307$	$n=8$	$t=4.4811$	$n=2$	$t=7.0269$	$n=4$
	p ; 0.10--0.05		p ; 0.025--0.0125		0.0025--0.0005	
	(1 / 5 ; 20%)		(2 / 2 ; 100%)		(3 / 3 ; 100%)	
II **	0.84 ± 0.13	0.81 ± 0.13	0.91 ± 0.12	0.81 ± 0.08	0.76	0.54
	$t=0.2307$	$n=2$	$t=1.3877$	$n=6$		
	p ; 0.45--0.40		p ; 0.15--0.10			
	(0 / 2 ; 0%)		(2 / 4 ; 50%)		(1 / 1 ; 100%)	

I *
II ** } Same as Table II

Finally, the effects of anti-sera on the migration inhibition test were investigated. In the preliminary experiment, anti-sera were put into the migration wells at two to four percent of the final concentration together with antigens, and were found to have marked feeding effects on the migration cells, as did the control sera. No specific effects was

Table VI Effects of Anti-sera to the Migration Inhibition of Mice Lymphoid Cells

migr. cells	anti-STL-1 lymphoid cells				
		un-treated		pretreated with normal sera	pretreated with anti-STL-1 sera
antigen	un-treated STL-1 cells	STL-1 cells pretreated with normal sera	STL-1 cells pretreaced with anti-STL-1 sera	un-treated STL-1 cells	un-treated STL-1 cells
Exp. No. 106	0.79 ± 0.15	0.79 ± 0.15	0.83 ± 0.12	0.84 ± 0.11	1.00 ± 0.10
	(0.84 ± 0.11)				
112	0.60 ± 0.11	0.78 ± 0.07	0.67 ± 0.08	0.92 ± 0.04	0.85 ± 0.02
	(0.97 ± 0.13)				
116	0.74 ± 0.06	0.78 ± 0.07	0.71 ± 0.06	0.83 ± 0.08	0.99 ± 0.08
	(0.99 ± 0.07)				

( ) : MII of non-sensitized control lymphoid cells with untreated STL-1 cells

observed. Therefore, in the present study each cell line was pretreated with 4% antisera at 37°C for 30 minutes. The treated cells were washed 3 times with PBS. Anti-STL-1 sera specifically affected the sensitized lymphoid cells, but not the target STL-1 cells in experiments No. 106 and 116 (Table VI).

Discussion

In migration inhibition tests, peritoneal macrophages and peripheral leukocytes have frequently been chosen as the migration cells.

In animal studies, guinea pigs, rats, mice and even chicken (VLAOVIC[29]) have been tested by this method and a correlation with in vivo delayed hypersensitivity has been proved. However when small rodents such as mice are used as the test system, many animals are needed to get enough peritoneal exsudate cells (AL-ASKARI[1]), even if a micro-method (CLAUSEN,1971[7], CLAUSEN,1975[8], HARRINGTON, 1973[12], FEDERLIN, 1971[9]) is available. An indirect migration inhibition test has also been devised (HOCHOVA, 1975[13]), but the procedure is time-consuming. Therefore, we considered it reasonable to use lymphoid tissue for the direct migration inhibition test, as POUPON[22], FIMMEL[10] and RANLØV[23] did. As mentioned before and also reported by RANLØV, the migration of mice spleen cells in the capillary technique is inconstant. We followed RANLØV's procedure in employing a 24 hours preincubation period in the migration inhibition test. Although this method gave a true indication of the specific migration inhibition, the standard deviations were large, from 50 to 70%, and thus the method was abondoned.

The spleen contains a lot of erythrocytes, and removal of them by NH_4Cl treatment is known to be inhibitory to migration, though NH_4Cl treatment has no effect on the cytotoxicity test. Hypotonic shock with distilled water is throught to be better than NH_4Cl treatment. However, slight contamination of intact erythrocytes does not inhibit the migration (Bendixen[5]), though a small amount of hemoglobin is very toxic to migration cells. Therefore, the thymus and lymph node cells were selected. An aging effect on the thymus was also noted by MEREDITH[20], but this disadvantage may be overcome by mixing of the lymph node cells. The migration area of the thymus was wider than that of the lymph node cells, but the difference in the sensitivity of thymus and lymph node cells in migration test was negligible (unpublished data).

Specific migration enhancement has been reported to occur at lower concentration of antigen (WEISBART[30], STEINER[26]). In our study, no case showed significant specific enhancement, since MIIs of sensitized lymphoid cells were both higher than 1.00 and significantly higher than MIIs of non-sensitized lymphoid cells. Most of the negative cases had MII values of sensitized lymphoid cells lower than 1.00, but higher than MII of non-sensitized lymphoid cells. WEISBART[30] viewed this reduction of migration inhibition as a sort of migration enhancement. But we consider it is more reasonable that such results derived from inappropriate immunization of the control animals, rather than a lower concentration of antigens. The optimal cell concentration was taken as 10,000 cells/ml, because antigenic

STL-1 cells started to show positive migration inhibition at 5000 cells/ml, and at more than 20,000 cells/ml they showed non-specific inhibition. Following the special classification, many negative results were found to be due to non-specific migration inhibition of non-sensitized lymphoid cells. Our St/a Fib. mice were obtained from the conventional minimal disease section, rather than the SPF or germ free unit. Nor was the migration inhibition test performed under special sterile conditions. Therefore, the control mice could have been sensitized by bacterial or other unknown antigens, and these unexpectedly sensitized control tissues could have reacted with corresponding antigens in the migration inhibition test. It is noteworthy that mice from the conventional unit had larger lymph nodes than ones from SPF unit.

In man there are reports on common reactivity and subclinical infections of normal persons (BODDIE, 1975[6]), in which the migration inhibition test was employed. The control lymphoid cells play an important role in the migration inhibition test, and pose more difficult problems in human than animals experiments. Nonspecific inhibition of non-sensitized lymphoid cells might partially explain the unreliability on the migration inhibition test mentioned by ARMISTEAD[3], ROSENBURG[25], and REMOLD[24]. Of course this is not the only possible explanation. TUBERGEN[28] noted MIF activity in the supernatant of normal fibroblast culture, and proposed that MIF was not a specific product of the antigenic response, and that it could be produced by normal cultivated cells in the S-phase. On the other hand, FOX[11] stated that fetal calf serum contains MIF and MSF (migration stimulatory factor). It is also noted that most of the neoplastic transformed cells have common antigenicities with foetal tissues. And multiparous females react to the transformed cells much more strongly than vergin females or male animals (BALDWIN, 1973[4]).

The effects of antiserum on the migration inhibition test have also been frequently reported. BENDIXEN[5] failed to demonstrate the effect in human materials with granulomatous infection and autoimmune disease. LACOUR[7] showed the increase of migration inhibition with addition of antisera. In constant, POUPON[22], MARABELL[19] and KIM[16] reported decreased migration inhibition with addition of antisera in mice plasmacytoma, human lung cancer and rat mammary tumor system, respectively.

Our experiments showed a specific effect on the effector cells without blocking the target cells. These data suggested that antigenic inhibition is based on the presence of free or antigen-excess antigen-antibody complex in the serum of sensitized animals. And they are keeping with POUPON's result that the serum from tumor-bearing mice inhibited the migration of sensitized lymphoid cells without addition of specific antigens.

Acknowledgement

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和文抄録

STL-1 細胞 (transformed mice fibroblast) を用いた細胞免疫反応における遊走阻止率の検討

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in vitro で自然に transform した ST/a mice の fibroblast STL-1 を用いて実験した．この細胞の性格は螢光抗体法，in vivo 移植実験などで，免疫学的に詳細に調べられており，今回は毛細管での遊走阻止反応を用いて再検討した．

その結果，遊走阻止反応においては，正常コントロールとして使われるリンパ球が，細菌その他の抗原によって感作され，これらが false negative result の一大要因をなすものと考えられた．

これらの不適当なコントロールを除外すると，遊走阻止反応は，STL-1 system においても，信頼できる免疫検査法であった．更に拡血清を用いた blocking 作用も，antigenic inhibition であることを，今回の実験で証明し得た．