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Specificity of Cell-mediated Transplantation Reactions

I. STUDIES WITH THE TECHNIQUE OF INHIBITION OF MIGRATION AND AN ASSAY OF TUMOUR IMMUNITY *IN VIVO*

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Summary. The *in vitro* assay of inhibition of leucocyte migration was used in two forms for the study of the specificity of transplantation immunity in mice. In one system thymus cells (antigen) were mixed with peritoneal exudate cells (PEC) from mice immunized with skin grafts and inhibition of migration was detected only when the antigenic cells were of donor origin or were from a third-party strain which shared an H-2 region (K or D) with the graft donor. However, in another form of the assay, in which sensitized lymph node cells (LNC) were mixed with antigenic PEC, inhibition of migration was detected also using antigenic cells from third-party strains which did not share an H-2 region with the donor and possessed only few H-2 specificities of the donor. The inhibition of migration mediated by LNC was abrogated with the use of anti-θ sera.

Transplantation immunity was studied *in vivo* with an assay of resistance to the growth of a third-party tumour (SaI) in mice pre-immunized with skin grafts from congenic strains having different H-2 antigens. Resistance was found in cases where the tumour cells did not share an H-2 region with the graft donor, but was weaker than the resistance obtained upon immunization with grafts from strains sharing an H-2 region with the tumour donor.

INTRODUCTION

The technique of inhibition of cell migration from capillary tubes by antigen has been used extensively for the *in vitro* study of diverse forms of cell-mediated immunity (David and David, 1972). The mechanism responsible for the phenomenon has been shown in most studies to be the inhibition of the migration of macrophages mediated by a soluble migration inhibitory factor (MIF) released from sensitized lymphocytes upon their interaction with specific antigen.

In vitro studies of cell-mediated transplantation immunity have been confined largely to methods involving metabolic alterations or destruction of target cells derived from the graft donor by sensitized lymphoid cells of the recipient; the assay of inhibition of migration has been adopted for transplantation studies only infrequently (Al-Askari, David, Lawrence and Thomas, 1965; Al-Askari and Lawrence, 1969; Friedman, 1971; Al-Askari and

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Lawrence, 1972). In an initial report (Lake, Sabbadini and Sehon, 1971) the inhibition of migration technique was considered to be a sensitive approach for the study of transplantation immunity in mice. In the present report the immunological specificity and relative sensitivity of two modifications of the assay are considered, and the results are compared with those obtained with techniques of cell-mediated cytotoxicity *in vitro* and studies of transplantation immunity *in vivo*.

MATERIALS AND METHODS

Animals

Inbred DBA/2J, C3H/HeJ, A/J, BALB/cJ, C57Bl/6J and congenic C57Bl/10, B10.D2 (new), B10.A, B10.BR, and B10.M mice were obtained from the Jackson Laboratory, Bar Harbour, Maine. Males were used in all aspects of the migration experiments, while females were used in the experiments of tumour growth.

Immunizations

Animals to be used as the source of sensitized peritoneal exudate cells (PEC) in the migration experiments, or those which served as pre-sensitized hosts in the tumour growth assays, were 10–14 weeks of age. Each received a single 1-cm^2 graft of non-active trunk skin on the lateral thoracic region according to the methods of Billingham and Medawar (1950). The grafts were secured with a spray plastic dressing (Aeroplast Dressing, Parke, Davis and Company, Detroit) and a transparent adhesive. Mice used as a source of sensitized lymph node cells (LNC) received bilateral skin grafts. In all experiments sensitized lymphoid cells were recovered on days 9–11 after grafting.

Tumour allografts

Sarcoma I (SaI), a rapidly growing spindle-cell tumour, was obtained from the Jackson Laboratory and maintained by serial passage in female A/J mice, the strain of tumour origin. Ascites tumour cells were aspirated aseptically with a syringe from host animals and were washed once in Hanks's balanced salt solution (HBSS) with centrifugation at 150 ***g*** (5 minutes) and resuspended in HBSS to produce a nucleated cell concentration of 10×10^6 cells/ml. Cell viability was in excess of 95 per cent as determined by the exclusion of 0·2 per cent Trypan Blue. In each experiment all groups of mice were inoculated subcutaneously on the dorsum with 2×10^6 tumour cells from a continuously mixed cell suspension and tumour growth was recorded as the mean of two perpendicular diameters.

Preparation of lymphoid cells

Thymuses and lymph nodes (axillary and brachial) were obtained by dissection using sterile procedures. The organs were teased into fragments in chilled HBSS in plastic Petri dishes (Falcon Plastics, Oxnard, California) with syringe needles and the cell suspensions were transferred to plastic tubes. Large fragments were allowed to settle for 5–10 minutes and the cell suspension was washed three times in HBSS as above. Cell concentrations were determined with Turk's solution.

Preparation of PEC

PEC were induced in both normal and skin-grafted mice by an intraperitoneal injection

of a solution of dextran, grade 2-P (Pharmachem, Bethlehem, Maryland). The dextran had a molecular weight of $5–40 \times 10^6$ and was dissolved at a concentration of 35·5 mg/ml in phosphate-buffered saline. After autoclaving, the solution was stored at −20°. Each animal received 1·5 ml of the solution 3 days prior to the recovery of the PEC. PEC were harvested from exsanguinated mice by injecting 6–7 ml of cold HBSS i.p., massaging the abdomen and aspirating the fluid with a syringe. The PEC suspension was washed three times in HBSS, a single cell suspension was decanted from cell aggregates and debris, and the cell concentration was determined as above.

Migration of PEC

The methods used were a modification of those described by David, Al-Askari, Lawrence and Thomas (1964). PEC (20×10^6) were dispensed into a series of sterile plastic centrifuge tubes. Thymus cells (5×10^6) or LNC in variable numbers, from appropriate strains, were added to some of these PEC suspensions, while other PEC suspensions, which served as one form of control, received no additional cells. The tubes were centrifuged at 150 ***g*** for 5 minutes and the cell pellet was resuspended in 0·45 ml of the tissue culture medium (TCM) comprising Medium 199 (Difco Laboratories, Detroit, Michigan), 20 per cent heated (56° for 30 minutes) foetal calf serum (Microbiological Associates, Bethesda, Maryland) and supplemented with 100 u/ml of penicillin and 100 μg/ml of streptomycin (Difco). This volume was adequate to fill seven capillary tubes. The cell suspensions were drawn into number 34502 capillary tubes (Kimax, Kimble Products, Owens, Illinois) which were sealed with a small high-temperature flame and maintained horizontally at 0–4° in sterile centrifuge tubes. The tubes were then centrifuged at 150 ***g*** for 5 minutes and placed in an ice bath. Tissue culture chambers were assembled from sterile disposable Petri dishes (Falcon Plastics) with lid number 3001 forming the cover and dish number 3002 containing a 6·25 cm^2 cover slip, fixed with a drop of silicone grease, forming the base. Capillaries (six per chamber) were cut 1 mm below the cell–fluid interface, mounted on the coverslip with silicone grease and two drops of TCM were applied to each tube mouth to avoid artifacts caused by evaporation. The lid was rapidly welded to the base at several points with a hot Pasteur pipette and the chamber was sealed with molten paraffin wax. TCM (6 ml) was slowly injected into the culture chamber via a temporary access channel. In some experiments Mackaness-type chambers containing two capillaries each and 2 ml of TCM were used. Following incubation of the chambers for 24 hours at 37°, migration areas were traced onto transparent film from dark-field images using a Nikon Model 6 Profile Projector (magnification ×20) and were integrated directly by planimetry. The percentage migration inhibition was calculated from the expression [(mean area of control − mean area of test migrations)/(mean area of control migrations)] × 100.

Studies with anti-theta serum

Anti-*θ* serum was prepared according to Reif and Allen (1964) in AKR mice, and used neat or diluted in HBSS (pH 7·2) containing 1·5 per cent heated foetal calf serum and 0·01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer. LNC ($1·5–2·0 \times 10^6$) were incubated for 30 minutes at 37° in antiserum, washed once with the above solution and resuspended in 1·0 ml of agarose-absorbed guinea-pig serum following the method of Cohen and Schlesinger (1970) (diluted 1:9) and incubated again for 30 minutes at 37°. The cells were washed once and resuspended in medium RPMI 1640 containing 20 per cent heat-inactivated foetal calf serum, 0·04 M HEPES and antibiotics

(100 U/ml of penicillin and 100 μg/ml of streptomycin) which also served as the medium for migration in these experiments. Some batches of anti-θ sera (diluted 1:2 in HBSS) were absorbed with A/J or AKR brain homogenates (1 volume serum: 5 volumes brain sediment for 1 hour at 4°. The antiserum was cytotoxic to 50 per cent of A/J thymus cells at a dilution of 1:256.

RESULTS

INHIBITION OF MIGRATION BY ANTIGENIC THYMOCYTES

The inhibition of migration of PEC obtained from mice immunized with skin grafts, by antigen in the form of thymus cells, was studied. Preliminary studies showed that the migration of PEC from normal mice was unaffected by including up to 60 per cent of

TABLE 1

REPRESENTATION OF PRESUMPTIVE IMMUNIZING ANTIGENS AND THOSE AVAILABLE FOR REACTION ON THIRD-PARTY CELLS

Donor	Recipient	Third party	H-2 specificities of the donor	
			H-2K	H-2D
C3H/HeJ (H-2^k)	C57BL/10 (H-2^b)		23* 1,3,8,11,25,45	32* 1,3
			Donor H-2 specificities presented by third patry	
		B10.BR (H-2^k)	23 1,3,8,11,25,45	32 1,3
		B10.A (H-2^a)	23 1,3,8,11,25,45	— –,3
		DBA/1 (H-2^q)	— 1,3,–,11,—,45	— –,3
		B10.D2, BALB/c (H-2^d)	— –,3,8,—,—,—	— –,3
A/J (H-2^a)	C57BL/10 (H-2^b)		23* 1,3,8,11,25,45	4* 3,13,41,42,43,44
			Donor H-2 specificities presented by third party	
		B10.A (H-2^a)	23 1,3,8,11,25,45	4 3,13,41,42,43,44
		B10.D2 (H-2^d)	— –,3,8,—,—,—	4 3,13,41,42,43,44
		B10.BR (H-2^k)	23 1,3,8,11,25,45	— 1,3,—,—,—,—,—
		DBA/1 (H-2^q)	— 1,3,–,11,—,45	— 3,13,—,—,43,—
		B10 M (H-2^f)	— –,–,8,—,—,—	— –,—,—,—,—,—
B10.M (H-2^f)	C57Bl/10 (H-2^b)		9* 8,37	9* 7
			Donor H-2 specificities presented by third party	
		SaI (H-2^a)	– 8,—	– –
C57BL/10 (H-2^b)	B10.D2 (H-2^d)		33* 5,39	2*
			Donor H-2 specificities presented by third party	
		SaI (H-2^a)	— 5,—	–

* A private H-2 specificity; a dash indicates that the specificity is not present (from the table of Klein and Shreffler, 1971).

syngeneic or allogeneic thymus cells in the migrating mixtures (Lake, 1972); however, the cell mixtures in the present study involve at most, the use of 20 per cent of thymus cells and 80 per cent PEC. PEC obtained from mice immunized with skin allografts 9–11 days earlier were markedly inhibited when mixed with thymus cells from the graft–donor strain. The reaction was shown to be immunologically specific since thymocytes from the recipient strain or from a third-party strain, unrelated to the graft donor, produced little or no inhibition (Lake *et al.*, 1971).

In the present experiments C57Bl/10 mice were grafted with skin from C3H/HeJ or A/J mice and tested for inhibition of migration with thymus cells from a panel of strains

having all, part, or none of the H-2 antigens of the graft donor strain as defined serologically (Table 1). In the first series of experiments PEC from C57Bl/10 mice grafted with C3H/HeJ skin were inhibited equally by C3H/HeJ and by B10.BR thymus cells (Table 2). Since B10.BR shares the same H-2 chromosome (according to the definition of Klein and Shreffler, 1971) with C3H/HeJ mice, but is otherwise genetically identical to the recipient C57Bl/10, this finding indicates that antigens determined by the H-2 complex are of paramount importance in this assay. Thymus cells from A/J caused strong inhibition of migration as well. This may have been expected, since all the antigens of C3H/HeJ

TABLE 2

SPECIFICITY OF INHIBITION OF MIGRATION OF C57Bl/10 PEC (IMMUNE TO C3H/HeJ) BY ANTIGENIC CELLS FROM DIFFERENT STRAINS

Experiment	Percentage inhibition of migration (±1 s.d.) induced by antigenic cells obtained from:					
	C3H/HeJ	B10.BR	A/J	BALB/c	DBA/1	C57Bl/10
1	30·4±6·9	30·2±6·7	25·3±8·9	9·9±6·0	12·7±6·1	———
2	44·5±2·6	48·9±5·5	40·1±6·2	19·3±4·1	2·0±7·3	6·9±5·4
3	37·9±3·4	37·8±1·4	43·3±2·1	15·0±7·8	9·4±7·7	13·2±2·3
4	49·0±5·0	46·8±3·7	38·7±6·3	5·4±9·6	−4·2±8·2	−4·3±8·0

PEC from the graft recipient were mixed in a ratio of 1:4 with thymus cells (antigen) from non-immunized mice prior to cell migration.

TABLE 3

SPECIFICITY OF INHIBITION OF MIGRATION OF C57Bl/10 PEC (IMMUNE TO A/J) BY ANTIGENIC CELLS FROM DIFFERENT STRAINS

Experiment	Percentage inhibition of migration (±1 s.d.) induced by antigenic cells obtained from:					
	A/J	B10.A	B10.BR	B10.D2	DBA/1	C57Bl/10
1	27·4±2·4	25·7±5·7	24·0±6·6	10·9±3·4	4·4±4·3	−3·2±2·7
2	33·5±5·6	27·3±1·9	23·2±7·2	17·6±10·3	5·9±7·0	−1·6±5·4
3	19·6±4·9	32·7±6·2	14·7±5·7	12·2±3·6	−1·9±10·9	7·8±4·0

PEC from the graft recipient were mixed in a ratio of 1:4 with thymus cells (antigen) from non-immunized mice prior to cell migration.

determined by the H-2K region are represented in strain A/J (Table 1). The absence of the D-region antigen H-2.32 of C3H/HeJ did not appear to reduce the inhibition. However, the addition of thymus cells from DBA/1 mice, which have four of the eight H-2 specificities of the graft donor, to the immune PEC resulted in migrations almost identical to those obtained in the presence of C57Bl/10 thymus cells, the syngeneic control. Thymus cells from BALB/c mice produced only minimal inhibitions in two experiments when compared to the effects of C57Bl/10 thymus cells.

In the second series of experiments, in which A/J was the graft donor, a similar pattern of results was obtained. The ability of B10.A thymus cells to produce inhibitions similar to those caused by cells from A/J mice (Table 3) confirms the dominance of H-2 antigens in this assay, as shown in the former group of experiments. Thymus cells from B10.BR and B10.D2, having all the K- and D-region H-2 antigens of A/J respectively (Table 1), produced inhibitions. However, as before, thymus cells from DBA/1 mice did not inhibit the migration of PEC from immune C57Bl/10 mice, although they carried six of the thirteen relevant H-2 specificities.

It is apparent from the design of the above migration technique that the sensitivity of the assay may be determined by two limiting parameters: the number of thymus cells in the migrating cell mixture; and the number of specifically sensitized cells in the peritoneal exudate population. The first possibility was excluded by the results of an experiment in which antigenic A/J thymus cells were added in varying numbers to PEC from C57Bl/6 mice grafted 10 days earlier with A/J skin. When A/J thymus cells constituted 20, 11, or 5·9 per cent of the migrating cell population, the degrees of inhibition of migration were identical (40 per cent inhibition). Only when the proportion of thymus cells was reduced to 2·0 per cent was decreased inhibition noted (23 per cent inhibition). However, an adjustment of the proportion of sensitized cells in the migrating cell mixture, to ensure their presence in non-limiting numbers, was only possible using the modification of the migration technique which follows.

INHIBITION OF MIGRATION BY SENSITIZED LNC

In this modification of the inhibition of migration assay, sensitized LNC, prepared from regional lymph nodes draining the graft site, were mixed with antigenic PEC. LNC from

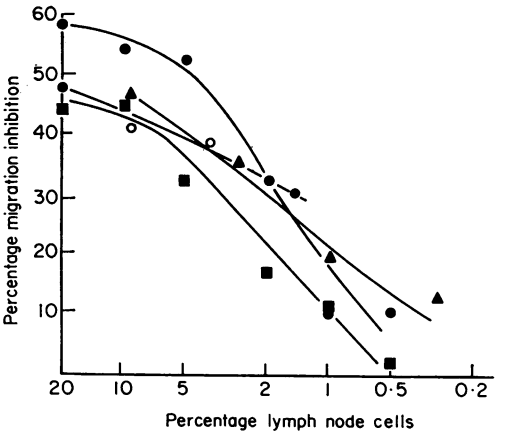

FIG. 1. Inhibition of migration of PEC of the graft donor upon mixing with sensitized LNC of the recipient. Effect of varying the numbers of LNC. The results from four experiments are shown in semi-logarithmic form.

normal allogeneic mice consistently showed no inhibitory activity when mixed with syngeneic or allogeneic PEC (Lake *et al.*, 1971; Lake, 1972). For preliminary studies LNC from A/J mice, grafted with C57Bl/6 skin 9–11 days earlier, were mixed in various proportions with antigenic PEC obtained from non-immunized C57Bl/6 mice. It is evident from the data shown on a semi-logarithmic plot (Fig. 1) that maximal inhibition was approached with a cell mixture containing as few as 5 per cent LNC, and that significant inhibition of migration could be obtained with as few as 2 per cent of sensitized LNC. It was therefore concluded that the use of 20 per cent of sensitized LNC in the migrating cell mixture should result in an assay having non-limiting numbers of sensitized cells.

In other experiments LNC were obtained from A/J mice at different intervals following skin grafting and assessed for the capacity to cause inhibition of migration. On the 6th day after grafting 20 and 11 per cent LNC respectively produced 33 and 22 per cent

inhibition of migration. Similarly on the 22nd day 20 and 10 per cent LNC respectively caused 26 and 7 per cent inhibition. These results contrast with the ability of LNC obtained 9–11 days following grafting to sustain near-maximum inhibitions at 10 per cent LNC and illustrate the suitability of the test to follow the kinetics of the immune response semi-quantitatively.

It was possible in the experiments of Tables 2 and 3 that thymus cells from strains DBA/1 and BALB/c failed to cause inhibition of migration because of low sensitivity of the test, perhaps due to limiting numbers of sensitized cells in the PEC. Therefore a third

TABLE 4

SPECIFICITY OF INHIBITION OF MIGRATION MEDIATED BY C57Bl/10 LNC (IMMUNE TO C3H/HeJ) UPON PEC FROM DIFFERENT STRAINS

Experiment	Percentage inhibition of migration (±1 s.d.) of PEC obtained from:					
	B10.BR	A/J	B10.A	B10.D2	DBA/1	C57Bl/10
1	44·3±7·2	48·8±6·3	———	16·2±4·9	23·9±10·0	14·1±4·7
2	34·8±3·0	———	———	27·4±4·0	25·5±4·9	11·3±4·5
3	47·5±5·7	———	41·6±3·5	14·7±5·3	50·7±6·7	7·9±7·4
4	———	31·5±3·7	———	8·4±6·0	18·0±8·2	−0·3±8·2

LNC from the graft recipient were mixed in a ratio of 1 : 4 with PEC (antigen) from non-immunized mice prior to cell migration.

TABLE 5

SPECIFICITY OF INHIBITION OF MIGRATION MEDIATED BY C57Bl/10 LNC (IMMUNE TO A/J) UPON PEC FROM DIFFERENT STRAINS

Experiment	Percentage inhibition of migration (±1 s.d.) of PEC obtained from:					
	B10.A	A/J	B10.BR	B10.D2	DBA/1	C57Bl/10
1	72·5±3·0	77·6±2·0	80·0±4·5	66·4±2·3	53·0±11·4	13·3±4·4
2	70·9±3·5	82·9±3·0	67·6±1·7	72·0±5·2	69·4±7·3	12·8±7·2
3	77·9±2·3	76·3±3·4	67·4±3·2	68·6±1·7	51·0±5·1	8·5±2·2

LNC from the graft recipient were mixed in a ratio of 1 : 4 with PEC (antigen) from non-immunized mice prior to cell migration.

group of experiments was performed using the above modification of the migration assay in an attempt to overcome this limitation. In these experiments LNC were obtained from C57Bl/10 mice immunized with C3H/HeJ skin grafts and the immune LNC were mixed with PEC from normal mice of several strains having all, part, or none of the H-2 antigens of the graft donor. Under these conditions the reactivity of the sensitized lymphocytes from C57Bl/10 against cells of B10.BR and A/J origin was confirmed (Table 4). Moreover, reactions were observed in every experiment against cells of both DBA/1 and B10.D2 origin which have some donor H-2 specificities but lack identity with either the K or D region of the H-2 chromosome of the donor (Table 1), which is in marked contrast to the negative or borderline reactions obtained in similar experiments using the previous migration system.

The fourth group of experiments (Table 5) confirmed these findings using the second donor–recipient combination. LNC from C57Bl/10 mice grafted with skin from strain A/J mice markedly inhibited the migrations of PEC from all allogeneic strains tested,

including PEC from DBA/1 mice, again in contrast with the results obtained in the previous assay using thymus cells as the source of antigen. In this experiment as well, the representation of donor H-2 specificities is incomplete on cells of DBA/1 mice; neither all the K- nor D-region antigens are present on DBA/1 cells (Table 1). PEC from C57Bl/10 mice were tested with the immune LNC in each experiment, and provide no evidence for non-specific inhibition of migration.

EFFECT OF ANTI-θ SERUM

To determine the cell type responsible for inhibition of migration mediated by LNC, sensitized LNC were obtained from A/J mice grafted 9–11 days earlier with C57Bl/6 skin and were treated with anti-θ serum and complement prior to mixing with antigenic

TABLE 6

ABROGATION OF LNC-MEDIATED INHIBITION OF MIGRATION WITH ANTI-θ SERUM AND COMPLEMENT

Experiment	LNC	Treatment of LNC		Inhibition of migration (per cent ± S.E.)
		Stage 1	Stage 2	
1	Immune	HBSS	HBSS	26·0±2·8
		Anti-θ 1:2, 0·4 ml	HBSS	30·6±2·4
		Anti-θ 1:2, 0·4 ml	Complement	7·0±3·7
		Anti-θ^a 1:2, 0·4 ml*	Complement	29·7±2·8
		Anti-θ^b 1:2, 0·4 ml†	Complement	19·3±1·8
	Normal	None	None	−8·4±4·7
		Anti-θ 1:2, 0·4 ml	Complement	−1·9±2·3
2	Immune	None	None	34·8±3·0
		NMS‡ neat, 0·5 ml	Complement	39·7±3·1
		Anti-θ neat, 0·5 ml	Complement	−1·0±3·4
		Anti-θ 1:2, 0·4 ml	Complement	18·6±2·6

PEC from C57Bl/6 mice were mixed with LNC from A/J mice which had been grafted with C57Bl/6 skin 9–11 days earlier. The LNC constituted 7 per cent of the migrating cells in experiment 1 and 9·1 per cent in experiment 2.

* Anti-θ^a serum was absorbed with A/J brain.
† Anti-θ^b serum was absorbed with AKR brain.
‡ NMS = normal mouse serum.

C57Bl/6 PEC (Table 6). From the data shown, and from other similar experiments, it is apparent that treatment of LNC with anti-θ serum and complement reduces or abolishes the ability of these cells to mediate inhibition of migration. The activity of the antiserum was removed upon prior absorption with A/J brain homogenates (A/J has the θ-C3H type) but was only slightly reduced upon absorption with AKR brain (possibly a dilution effect).

EXPERIMENTS *in vivo*

The observations of *in vitro* inhibition of migration reactions evoked by third-party cells not having identity with either of the two H-2 regions of the donor, suggested that a sensitive and quantitative assay for transplantation immunity *in vivo* might reveal similar reactions. Since prior grafting with A/J skin prevents growth of the A/J tumour SaI in most

allogeneic mice, skin grafts from other strains of mice sharing transplantation antigens in common with A/J should suppress tumour growth as well. Therefore mice were immunized with skin grafts from congenic strains (i.e. immunizations confined to H-2 antigens) and after 10 days were inoculated with the tumour. Donors were selected so that in some congenic donor–recipient combinations A/J would lack identity with the primary graft donor at both H-2 regions. It is evident (Table 7) that the growth of SaI in mice which had received syngeneic grafts was essentially identical to that in untreated hosts, and that prior immunization of C57Bl/10 mice with A/J skin grafts resulted in animals refractory to SaI growth. Skin grafts from B10.BR prevented tumour growth as well, and

TABLE 7

GROWTH OF SaI (H-2^a) IN MICE PRESENSITIZED TO DIFFERENT H-2 ANTIGENS

Recipient	Primary donor	Takes per number injected	Mean take size* (mm ± s.d.)	H–2 specificities of primary donor x indicates specificities in common with SaI
C57Bl/10 (H-2^b)				
	None	5/5	7·7 ± 4·4	
	C57Bl/10 (H-2^b)	5/5	9·2 ± 1·8	None
	B10.M (H-2^f)	2/5	5·0 ± 2·8	7, 8x, 37, 9
	B10.BR (H-2^k)	0/6	———	1x, 3x, 8x, 11x, 25x, 45x, 23x, 32
	A/J (H-2^a)	0/4	———	1x, 3x, 8x, 11x, 25x, 45x, 23x, 13x, 41x, 42x, 43x, 44x, 4x
B10.D2 (H–2^d)				
	None	7/7	3·6 ± 1·8	
	B10.D2 (H-2^d)	8/9†	3·4 ± 1·2	None
	C57Bl/10 (H-2^b)	3/8	2·6 ± 1·6	5x, 39, 33, 2
	B10.M (H-2^f)	4/7	1·0 ± 0·0	9,37,7
	A/J (H-2^a)	0/8	———	1x, 5x, 11x, 25x, 45x, 23x

* Growth attained by SaI on day 7 following s.c. inoculation of 2×10^6 tumour cells. Mean size of takes ± s.d. were calculated from the means of two perpendicular measurements.

† 9/9 on day 8.

this is attributed to the fact that the H-2K region of B10.BR is identical to the H-2K region of A/J (Table 1). Moreover, grafts from B10.M mice reduced tumour growth and incidence, which is notable since in this donor–recipient combination, sharing of a common H-2 region with the B10.M graft donor by the tumour does not occur, and only one of four of the donor H-2 specificities is represented on cells of SaI (H-2·8).

In a second experiment of similar design, B10.D2 mice served as tumour hosts (Table 7). As before, tumour growth was similar in non-treated mice and in those which had received syngeneic grafts 10 days prior to tumour inoculation, and skin grafts from A/J mice suppressed tumour growth entirely. Grafts from C57Bl/10 mice caused a reduction in tumour size when compared with controls and prevented tumour growth in about one-half of the recipients. Neither the H-2K nor H-2D region of C57Bl/10 is represented on cells of A/J, and only one of four donor H-2 specificities (H-2·5) is shared (Table 1). Similarly, skin grafts from B10.M mice caused a reduction of tumour growth. As noted for C57Bl/10, B10.M does not have identity with either of the two H-2 regions of A/J moreover, B10.M shares no known serologically defined H-2 specificities with A/J. Thus, in three donor–recipient combinations, resistance to the growth of a third-party tumour was afforded by a primary immunization involving only H-2 antigens and in the absence of shared H-2K or H-2D regions by donor and third-party cells.

DISCUSSION

In the present report the specificity of transplantation immunity *in vitro* was studied with two modifications of the migration assay. In the first modification the migrating cells consisted of a mixture of sensitized PEC from skin-grafted mice and thymus cells (antigen) from mice of the donor or third-party strains. In all the experiments of this design, PEC from the graft recipients were inhibited in their migration when mixed in ratio of 4:1 with thymus cells of the donor. The use of thymus cells from third-party strains, showed that the third-party strain required identity with at least one of the two regions of the H-2 complex of the donor (i.e. either K or D) to produce detectable inhibition of migration. These results, which indicate the presence of at least two distinct H-2 antigen systems in the migration assay, are in keeping with the molecular independence of H-2D- and H-2K-region antigens (Neauport-Sautes, Silvestre, Lilly and Kourilsky, 1973). Thymus cells from strains not having identity with the donor at either of the H-2 regions caused marginal or no inhibition.

In some of the present experiments, however, PEC from C57Bl/10 mice immune to C3H/HeJ appeared to react weakly toward thymus cells of BALB/c mice which lack identity with either H-2 region of the donor and have only two of the eight relevant donor H-2 specificities. As an alternative hypothesis, therefore, it is possible that the sensitivity of the assay was limited, and that the reasons for the failure to detect definitive inhibitions with other third-party antigens may have been quantitative. Although antigenic thymus cells were shown to be present in non-limiting numbers in this assay, it was possible that the numbers of specifically sensitized cells in the PEC were limiting. A second modification of the migration assay in which regional LNC from skin-grafted mice were mixed with antigenic PEC from normal mice, ensured the presence of sensitized cells in high numbers.

In the first experiment of this design LNC from C57Bl/10 mice immune to C3H/HeJ inhibited the migration of PEC of donor H-2 type similar to the results of the previous assay. Moreover, the LNC inhibited the migration of PEC from B10.D2 having only two of the eight relevant donor H-2 specificities and also inhibited the migration of PEC of DBA/1 mice having four of the eight donor H-2 specificities. In both cases neither donor H-2 region is shared by the third-party strains.

In the second series of these experiments, reactions to third-party cells were observed as well. Thus, LNC from C57Bl/10 mice immune to A/J inhibited the migration of PEC of DBA/1 which lacks identity with both H-2 regions of the donor and has only six of the thirteen relevant donor H-2 specificities.

Although quantitative comparisons of the inhibitions obtained using donor PEC with the inhibitions obtained using third-party strains were not attempted, the data represented in Fig. 1 show that only a large decrease in numbers of LNC resulted in reduced inhibitions and therefore indicate that the reaction of sensitized LNC, on a cell-for-cell basis, was considerably less potent against antigenic PEC lacking complete identity with either of the two H-2 regions of the donor than against donor-type PEC (Tables 4 and 5). Participation of non-H-2 antigens can be excluded in the response to the congenic strain B10.D2. However, reactions to non-H-2 antigens cannot be excluded in the reaction of LNC to cells to DBA/1, but are unlikely since attempts to demonstrate non-H-2 immunity (immunizations of the type: 129/J→C57Bl/6 and the reciprocal) using recipient LNC and donor PEC were not successful (unpublished results).

In conclusion, with a modification of the migration assay involving a mixture of sen-

sitized LNC and antigenic PEC, inhibition of migration was detected in all experiments with antigenic PEC from all third-party strains lacking identity with either H-2 region of the donor and sharing only a few public donor H-2 specificities. However, the reactions were weaker than those caused by donor antigens.

It is unknown if the mechanisms of the two forms of the migration assay presented in this paper are identical; however, two similar assays for cellular immunity in the guinea-pig, the first involving the use of immune PEC (Bloom and Bennett, 1966) and the second involving a mixture of normal PEC and immune LNC (David, 1966) were both shown to have MIF-dependent mechanisms. Further, Al-Askari and Lawrence (1972) have demonstrated that MIF but not allo-antibody is produced by cells from immune PEC using an experimental protocol very similar to that described here, which suggests that the inhibitions obtained in the first assay (immune PEC plus antigenic thymocytes) are mediated by MIF. In the second migration assay (immune LNC plus antigenic, normal PEC) the dependence of inhibition of migration upon θ-bearing cells is also not compatible with a conventional antibody-mediated mechanism. The possibility of cell-to-cell cytotoxicity among the migrating cells cannot account for the observed inhibitions since the ratios of cytotoxic cells to target cells, in both forms of the migration assay, were of the order of 100-fold less than required to evidence minimal cytotoxicity in the ^{51}Cr assay (unpublished results). The weight of evidence thus suggests that the two migration assays are similar in mechanism.

Al-Askari and Lawrence (1972) demonstrated the production of an MIF-like material by lymphocytes from skin-grafted mice which parallels the earlier findings of MIF-mediated inhibition of migration in guinea-pigs with cellular immunity (Bloom and Bennett, 1966). In view of the selective cytotoxicity of anti-theta serum to lymphoid cells of thymus-derived lineage (Raff, 1969), the abrogation of the capacity of sensitized LNC to inhibit migration following treatment with this antiserum provides direct confirmation for the role of T-cell-mediated immunity in this *in vitro* transplantation reaction.

It is of interest to note that with similar donor, recipient and third-party strains, absence of cross-reactivity was concluded from results using the method of *in vitro* cell-mediated cytotoxicity (Brondz and Golberg, 1970). Such conclusions are in apparent agreement with the results obtained with the first modification of the migration assay, but are at variance with the results obtained with the second, and presumably more sensitive modification.

The reactivity of sensitized cells to third-party antigens was confirmed by *in vivo* transplantation experiments in which the growth of the tumour, SaI was suppressed in mice pre-immunized with skin grafts. B10.D2 mice grafted with C57Bl/10 skin were resistant to SaI tumour growth and C57Bl/10 mice grafted with B10.M skin were resistant as well. In both cases the third-party strain, A/J (SaI), lacked identity with either H-2 region of the graft donor and shared only one H-2 specificity with the donor. In addition, B10.M skin grafts conferred some resistance to challenge with SaI in B10.D2 mice as well, despite the lack of identity with either of the donor H-2 regions as in the two previous cases, and the absence of known, shared H-2 specificities with A/J. These unexpected results were confirmed in an *in vitro* cytotoxicity assay (Lake, Sabbadini and Sehon, 1974) and indicate either that the available serological information is incomplete or that some H-2 antigens evoke cell-mediated reactions but little or no antibody. Since congenic mice were used for immunization, tumour resistance can be attributed only to immunity directed at antigens determined by genes very near or at the H-2 complex. Other studies *in vivo* using skin-grafting techniques (Berrian and Jacobs, 1959; Klein and Murphy, 1973) are consistent with these results.

It is pertinent to note that in all third-party combinations, some tumour growth did occur, which suggests that, although detectable, the immunity was quite weak. This aspect could be less apparent with skin grafting techniques.

The resistance to the growth of SaI *in vivo* in skin-grafted mice may be attributed to cellular immunity since immunological enhancement of SaI, not resistance, is the usual consequence of the presence of iso-antibody (Gorer and Kaliss, 1959).

The results in this report contrast with the conclusions of Brondz (1968) and Brondz and Golberg (1970) obtained with the method of *in vivo* cell-mediated cytotoxicity and with similar observations by others (Ginsburg, 1968; Mauel, Rudolf, Chapuis and Brunner, 1970; Ax, Koren and Fischer, 1971; Berke and Levey, 1972). In all of these studies very little or no immunity was detected against target cells from any third-party strains not sharing a donor H-2 region. As indicated previously, the reactions described in this report both *in vitro* and *in vivo* to third-party cells lacking H-2 region identity with the donor were demonstrably weaker than the reactions towards donor-type target cells. These quantitative differences formed the basis of a study of the specificity of target cell destruction in which the existence of sensitized cells, cytotoxic to third-party target cells, was demonstrated (Lake *et al.*, 1974).

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