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COMPARISON BETWEEN THE DIRECT
CAPILLARY TUBE LEUCOCYTE MIGRATION
TECHNIQUE (LMCT) AND THE DIRECT
AGAROSE LEUCOCYTE MIGRATION
TECHNIQUE (LMAT) AS INDICATORS OF
TUMOUR-DIRECTED CELL-MEDIATED
HYPERSENSITIVITY (TCMH) IN PATIENTS
WITH RENAL CARCINOMA

By

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In 1971 Clausen (8) introduced the leucocyte migration technique in agarose (LMAT) as an *in vitro* correlate of cellular hypersensitivity (CH) to PPD. This technique was a further development of the capillary tube leucocyte migration technique (LMCT) originally described by Bendixen & Søborg (2). Using PPD-concentrations from 1.6–400 $\mu\text{g/ml}$ Clausen found a dose-related effect on leucocytes from Mantoux positive persons correlating with skin-reactivity to PPD (10). Comparing LMAT and LMCT, LMAT was found to be more sensitive than LMCT in demonstrating CH to PPD (9). Since then, conflicting results have been reported concerning the ability of LMAT to detect CH to PPD (1, 4, 16). However, great variability in PPD preparations and concentrations in the different investigations are evident.

Tissue extracts as antigen in LMAT have been the subject of very few studies (3, 5, 6, 7). In recent LMCT studies by

Kjaer (11, 12, 13) with crude tumour extracts as antigen in patients with renal carcinoma, it was found that: 1) a specific dose-related TCMH could be detected using extracts of allogeneic hypernephroma tissue (12); and 2) a clear toxic effect of tumour extract could be found in both patients and controls at 600 mg of protein/ml (13).

In its present modification LMAT possesses some advantages over LMCT: LMAT uses 50 per cent of the cells needed in LMCT, LMAT saves antigen, and LMAT is less time-consuming than LMCT. For monitoring TCMH in cancer patients on small volumes of blood and tumour antigen LMAT seems an obvious possibility. We therefore decided to compare LMCT and LMAT using crude tumour extracts at concentrations showing specific reactivity in LMCT.

MATERIALS AND METHODS

A total of 19 persons divided into the following groups were investigated:

Five consecutive patients with histologically verified hypernephroma (three men and two women, aged 37–67 years). The clinical data are presented in Table 1.

Fourteen healthy blood donors from the blood donor group of Rigshospitalet, Copenhagen (nine men and five women, aged 22–76 years): nine of these (seven men and two women) were tested as control patients in the LMAT and five (two men and three women) served as controls in the LMCT.

Preparation of Tissue Extracts

After removal of the kidney, specimens were obtained from tumour tissue and from normal tissue not macroscopically invaded by tumour. This was performed aseptically, and always within 30 min after removal of the kidney. The specimens were kept at -20°C . In the following procedure, tissue from each renal carcinoma and corresponding normal renal parenchyma was kept and treated separately. No pooling was performed.

On the day of operation or on the day after, the specimens were thawed, cut into small pieces, suspended in Hanks' solution, and homogenized in a M.S.E. blade homogenizer at 15,000 rev/min for 7 min. The homogenates were kept at 4°C overnight, and then centrifuged at 1000 rev/min for 20 min.

The protein concentration of the supernatant was determined by Lowry's method, and adjusted to 1 mg/ml by dilution with Hanks' medium. Antibiotics were added: penicillin G, 1500 i.u./ml, and streptomycin, 1500 $\mu\text{g}/\text{ml}$. Culture reports before addition of antibiotics showed that all extracts were sterile. $2\frac{1}{2}$ -ml

TABLE I
Five Patients with Hypernephroma. Clinical Data.

Patient no.	Sex	Age	Metastases	
			local	distant
1	male	37	—	+
2	female	67	—	—
3	male	58	—	+
4	female	55	+	—
5	male	62	—	+

aliquots of the extracts were transferred to sterile glass ampoules and lyophilized. Immediately before use in the test the extracts were dissolved in sterile water.

Leucocyte Suspension

Peripheral venous blood was collected from a cubital vein in 10-ml polystyrol tubes containing 250 i.u. of heparin without preservatives and 2.0 ml of dextran 250. The tubes were carefully inverted 10 times, and placed for 1 h at 37° C. After sedimentation of the erythrocytes, the leucocyte-rich supernatant was transferred to polyethylene tubes, and centrifuged at 1000 *g* for 5 min. The leucocytes were washed six times in Hanks' medium and resuspended in TC 199 with 10 per cent horse serum to a concentration of 2.2×10^8 cells/ml.

LMCT

The LMCT was performed as described by Bendixen & Søbørg (2), but micromodified according to Maini et al. (15) in the following way:

The leucocyte suspension was collected in a suitable number of 20- μ l glass capillary tubes, each capillary tube containing approximately 3×10^6 leucocytes. After sealing one end in a flame and centrifuging at 2000 rev/min for 10 min, the capillary tubes were cut just below the cell-fluid interphase, and fixed with a drop of silicone grease on the bottom of a 0.5-ml disposable polystyrene culture chamber containing TC 199 and 10 per cent horse serum. The tissue extract was added, and the chamber was sealed with a microscope coverslip. Four culture chambers were set up for each concentration of tissue extract employed. Within a set of identical cultures the variation from one migration area to another did not exceed 5 per cent.

After 24 h at 37° C, the migration areas of leucocyte around the openings of the capillary tubes were studied in a projection microscope, drawn on paper, cut, and weighed. The average area of the antigen-containing cultures (M_x) and control cultures (M_o), containing only TC 199 + 10 per cent horse serum, from the same blood sample determine the migration index *M*: $MI = M_x/M_o$.

A MI of less than 1.0 indicates an inhibition, and a MI of more than 1.0, a stimulation of leucocyte migration.

LMAT

The LMAT was performed as described in detail by Clausen (8): Fresh agarose medium was prepared every day. The agarose medium contained 1 per cent agarose, 10 per cent horse serum, 66 i.u. penicillin, and 66 μg streptomycin/ml. Sodium bicarbonate was added to adjust the pH to between 7.2 and 7.4 in 2 per cent CO_2 in air. The 47°C agarose-serum-TC 199 medium was poured into plastic petri dishes, 5 ml in each, and allowed to cool. After the gel had formed, wells, 2.3 mm in diameter, were punched out in the agarose medium.

The leucocyte suspension prepared as described above was divided into 90 μl aliquots. To these were added varying amounts of tissue extracts in final concentrations of 10, 50, 100, 300, 400, and 600 μg protein/ml. In control suspensions no tissue extract was added. After incubation for 30 min at 37°C , 7- μl portions of each cell suspension were placed in the wells in the agarose medium. Three to four cultures containing 1.5×10^6 leucocytes each were made from each leucocyte suspension. The agarose plates were then incubated at 37°C for 24 h in 2 per cent CO_2 , 98 per cent atmospheric saturated with water vapour. After 24 h the migration areas were studied in a projection microscope, drawn, and measured by planimetry.

Calculations of MI were performed as described under LMCT.

Test Protocol

In the patients with hypernephroma two tests were performed, if possible both in the preoperative period. Blood donors were tested only once. Systematic titration experiments with allogeneic hypernephroma and normal renal tissue extracts in the LMCT using extract concentrations of 10, 50, 100, 300, 400, and 600 μg protein/ml have been previously reported (12, 13). The results indicated a specifically altered, dose-related leucocyte reactivity in patients with hypernephroma without distant metastases manifest at concentrations of 50, 100, 300, and 400 μg protein/ml.

In renal carcinoma patients with distant metastases, an increased leucocyte reactivity in the LMCT by using six or 10 leucocyte washings instead of three has been shown earlier (13), and great variability of individual tumour extracts to induce leucocyte migration inhibition in patients with non-metastatic renal carcinoma has been found (14).

For these reasons, in the present study six leucocyte washings were used in both LMCT and LMAT, as some patients with distant metastases were included in the series investigated. Moreover, only highly antigenic tumour extracts (MIs of more than 20 per cent (14)) were used. Because of limitations in the available amount of blood for each test, the comparison between LMCT and

LMAT was performed over 2 days, involving either tumour extract or corresponding normal renal tissue extract in both techniques at concentrations of 10, 50, 100, 300, 400, and 600 μg protein/ml. Likewise the total volume of blood available for one test in a blood donor was 30 ml. Therefore each blood donor served as control for either tumour extract or corresponding normal renal tissue extract in either LMCT or LMAT.

Statistical Evaluation

After completing the series of patients and controls, individual titration curves were drawn. Groups of results were compared at fixed antigen concentrations by means of a computerized Wilcoxon Mann-Whitney test. Level of significance = 5 per cent.

RESULTS

The results of the comparison between LMCT and LMAT are shown in Table 2. Individual titration curves from the controls and the five patients with renal carcinoma are given in Figs. 1–5.

In blood donors a clear inhibitory effect of both allogeneic hypernephroma and corresponding normal renal tissue was found in the LMCT at concentrations of 400 and 600 $\mu\text{g}/\text{ml}$ (Fig. 1). In the LMAT, inhibition was manifest at 400 and 600 $\mu\text{g}/\text{ml}$ for allogeneic hypernephroma, and at 600 $\mu\text{g}/\text{ml}$ for normal renal tissue (Fig. 2).

In patients with hypernephroma, inhibition in the LMCT was manifest at 300, 400, and 600 $\mu\text{g}/\text{ml}$ for allogeneic hypernephroma, and at 400 and 600 $\mu\text{g}/\text{ml}$ for allogeneic renal tissue. In the LMAT, only 600 $\mu\text{g}/\text{ml}$ caused inhibition.

Comparing reactivity towards allogeneic hypernephroma and towards allogeneic renal tissue, no significant differences were found in the blood donors between allogeneic hypernephroma and renal tissue in either LMCT or LMAT at any concentration employed. However, patients with hypernephroma showed an altered reactivity in the LMCT towards extracts of allogeneic hypernephroma at concentrations of 300, 400, and 600 $\mu\text{g}/\text{ml}$, but not in the LMAT (Figs. 3–5).

Comparing differences in the techniques, LMCT and LMAT, a significant inhibition of blood donor leucocyte mi-

gration was seen in the LMCT at 600 $\mu\text{g}/\text{ml}$ using both allogeneic hypernephroma and renal tissue (Figs. 1-2). In hypernephroma patients a significant migration inhibition using allogeneic hypernephroma in LMCT was manifest at 300, 400, and 600 $\mu\text{g}/\text{ml}$ compared with LMAT, and at 400 and 600 $\mu\text{g}/\text{ml}$ using allogeneic renal tissue (Figs. 3-5).

TABLE
Comparison between

Blood Donors, LMCT

Exp. No.	Allogeneic Hypernephroma Tissue Extract ($\mu\text{g}/\text{ml}$)					
	A 10	B 50	C 100	D 300	E 400	F 600
MI						
1	0.88	1.17	1.17	0.95	0.91	0.67
2	1.01	0.96	0.94	0.88	0.85	0.49
3	0.99	1.02	0.94	0.85	0.82	0.33
4	1.19	1.06	1.12	0.96	0.88	0.51
5	0.95	0.90	0.97	0.83	0.83	0.48

Wilcoxon Mann-Whitney test (LMCT):

A-B, $P > 0.05$; A-C, $P > 0.05$; A-D, $P > 0.05$; A-E, $P < 0.05$; A-F, $P < 0.01$.
G-H, $P > 0.05$; G-I, $P > 0.05$; G-J, $P < 0.05$; G-K, $P < 0.01$; G-L, $P < 0.01$.

Blood Donors, LMAT

Exp. No.	Allogeneic Hypernephroma Tissue Extract ($\mu\text{g}/\text{ml}$)					
	A 10	B 50	C 100	D 300	E 400	F 600
MI						
1	0.96	0.97	0.93	0.93	0.91	0.87
2	0.99	1.01	0.99	0.94	0.89	0.82
3	0.97	0.96	1.00	0.98	0.86	0.77
4	0.98	0.99	0.98	0.92	0.85	0.69
5	1.02	1.01	1.01	0.98	0.96	0.95

Wilcoxon Mann-Whitney test (LMAT):

A-B, $P > 0.05$; A-C, $P > 0.05$; A-D, $P > 0.05$; A-E, $P < 0.05$; A-F, $P < 0.01$.
G-H, $P > 0.05$; G-I, $P > 0.05$; G-J, $P > 0.05$; G-K, $P > 0.05$; G-L, $P < 0.05$.

The results of the present study seem to indicate that a state of altered reactivity of leucocytes from patients with renal carcinoma towards allogeneic hypernephroma tissue extract can be detected in the LMCT but not in the LMAT. The reactivity is manifest at concentrations of 300 and 400 μg protein/ml, which is consistent with earlier findings (12,

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LMCT and LMAT.

Blood Donors, LMCT

Exp. No.	Allogeneic Normal Renal Tissue Extract ($\mu\text{g}/\text{ml}$)					
	G 10	H 50	I 100	J 300	K 400	L 600
MI						
1	1.10	1.04	1.02	0.84	0.82	0.70
2	0.96	0.93	0.90	0.96	0.87	0.56
3	1.09	1.12	1.04	0.97	0.89	0.40
4	1.09	0.98	0.95	0.90	0.88	0.35
5	0.97	1.02	0.95	0.88	0.85	0.46

Wilcoxon Mann-Whitney test (LMCT):

A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P > 0.05$; E-K, $P > 0.05$; F-L, $P > 0.05$.

Blood Donors, LMAT

Exp. No.	Allogeneic Normal Renal Tissue Extract ($\mu\text{g}/\text{ml}$)					
	G 10	H 50	I 100	J 300	K 400	L 600
MI						
1	1.10	1.03	1.04	1.03	1.03	1.00
2	1.00	0.99	0.99	0.97	0.99	0.91
3	1.03	1.02	0.98	0.98	0.95	0.89
4	0.99	0.96	0.93	0.93	0.85	0.72
5	1.00	1.01	1.01	0.98	0.96	0.95

Wilcoxon Mann-Whitney test (LMAT):

A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P > 0.05$; E-K, $P > 0.05$; F-L, $P > 0.05$.

TABLE

Hypernephroma Patients, LMCT

Allogeneic Hypernephroma Tissue Extract (μg/ml)						
Exp.	A	B	C	D	E	F
No.	10	50	100	300	400	600
MI						
1	0.85	0.93	0.81	0.69	0.56	0.45
2	1.04	0.99	0.95	0.82	0.68	0.56
3	1.08	0.92	0.96	0.72	0.60	0.51
4	1.10	1.03	1.11	0.73	0.68	0.54
5	0.80	0.85	0.71	0.58	0.60	0.49

Wilcoxon Mann-Whitney test (LMCT):

A-B, $P > 0.05$; A-C, $P > 0.05$; A-D, $P < 0.05$; A-E, $P < 0.01$; A-F, $P < 0.01$.
 G-H, $P > 0.05$; G-I, $P > 0.05$; G-J, $P > 0.05$; G-K, $P < 0.05$; G-L, $P < 0.01$.

Hypernephroma Patients, LMAT

Allogeneic Hypernephroma Tissue Extract ($\mu\text{g/ml}$)						
Exp.	A	B	C	D	E	F
No.	10	50	100	300	400	600
MI						
1	1.12	1.11	1.15	1.05	0.99	0.92
2	1.01	1.03	0.98	0.95	0.91	0.91
3	0.98	1.01	1.07	1.06	1.05	1.00
4	0.95	0.94	0.93	1.04	1.02	0.97
5	1.04	1.03	1.08	1.00	1.02	0.93

Wilcoxon Mann-Whitney test (LMAT):

A-B, $P > 0.05$; A-C, $P > 0.05$; A-D, $P > 0.05$; A-E, $P > 0.05$; A-F, $P > 0.05$.
 G-H, $P > 0.05$; G-I, $P > 0.05$; G-J, $P > 0.05$; G-K, $P > 0.05$; G-L, $P < 0.05$.

Comparison between Techniques,

LMCT-LMAT, hypernephroma patients

A-A, $P > 0.05$; B-B, $P > 0.05$; C-C, $P > 0.05$; D-D, $P < 0.01$; E-E, $P < 0.01$;
 F-F, $P < 0.01$.

G-G, $P > 0.05$; H-H, $P > 0.05$; I-I, $P > 0.05$; J-J, $P > 0.05$; K-K, $P < 0.05$;
 L-L, $P < 0.05$.

2 (continued)

Hypernephroma Patients, LMCT

Exp. No.	Allogeneic Normal Renal Tissue Extract ($\mu\text{g/ml}$)					
	G 10	H 50	I 100	J 300	K 400	L 600
MI						
1	0.88	0.90	0.95	0.85	0.87	0.80
2	1.10	0.95	1.07	0.98	0.85	0.81
3	1.15	1.08	1.07	0.92	0.89	0.58
4	0.95	0.93	0.92	0.88	0.85	0.57
5	1.07	1.04	1.01	0.92	0.83	0.64

Wilcoxon Mann-Whitney test (LMCT):

A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P < 0.01$; E-K, $P < 0.01$;
 F-L, $P < 0.01$.

Hypernephroma Patients, LMAT

Exp. No.	Allogeneic Normal Renal Tissue Extract ($\mu\text{g/ml}$)					
	G 10	H 50	I 100	J 300	K 400	L 600
MI						
1	1.00	0.95	0.97	0.94	0.93	0.87
2	0.99	1.01	1.01	1.01	1.01	0.98
3	0.98	0.92	0.92	0.96	0.98	0.95
4	1.06	0.98	1.02	0.95	0.94	0.91
5	0.97	0.95	0.94	0.90	0.88	0.73

Wilcoxon Mann-Whitney test (LMAT):

A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P > 0.05$; E-K, $P > 0.05$;
 F-L, $P > 0.05$.

Wilcoxon Mann-Whitney Test:

LMCT-LMAT, blood donors

A-A, $P > 0.05$; B-B, $P > 0.05$; C-C, $P > 0.05$; D-D, $P > 0.05$; E-E, $P > 0.05$;
 F-F, $P < 0.01$.

G-G, $P > 0.05$; H-H, $P > 0.05$; I-I, $P > 0.05$; J-J, $P > 0.05$; K-K, $P > 0.05$;
 L-L, $P < 0.01$.

LMCT, hypernephroma patients, LMCT, blood donors

A-A, $P > 0.05$; B-B, $P > 0.05$; C-C, $P > 0.05$; D-D, $P < 0.01$; E-E, $P < 0.01$; F-F, $P > 0.05$.

A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P < 0.01$; E-K, $P < 0.01$; F-L, $P > 0.05$.

13). Moreover, a significantly greater effect of both allogeneic hypernephroma and renal tissue is present in the LMCT than in the LMAT using 600 $\mu\text{g/ml}$.

DISCUSSION

To our knowledge comparisons between LMCT and LMAT using crude tissue extracts as antigens have not been reported earlier. Such a comparison is of major interest, since one important practical use of LMAT in clinical immunology depends on its ability to detect cellular hypersensitivity to tissue antigens.

Using LMAT and encephalitogenic protein prepared from bovine brain tissue as antigen, Bergstrand et al. (5) reported significant reactivity in 16 out of 24 patients with multiple sclerosis. The concentrations employed ranged from 10–250 $\mu\text{g/ml}$. Reactivity was found at 50 and 250 $\mu\text{g/ml}$ in controls. Significant reactivity in the patients was manifest at 10, 50, and 250 $\mu\text{g/ml}$. In another study (6) the effect of low-dose encephalitogenic protein (0.03–0.3 $\mu\text{g/ml}$) was investigated in the same group of patients. Migration indices tended to be higher in patients with multiple sclerosis than in controls.

The results from the present investigation cannot be compared with those of Bergstrand et al. because of differences in antigen preparation. With serologically-defined antigens prepared from human spleens Bendixen et al. (3) found some reactivity in kidney-transplanted patients using 50 $\mu\text{g protein/ml}$ in the LMAT. No titration experiments were reported.

Boddie et al. (7) used KCl-extracts of human lung car-

2 (continued)

 LMAT, hypernephroma patients, LMAT, blood donors

 A-A, $P > 0.05$; B-B, $P > 0.05$; C-C, $P > 0.05$; D-D, $P < 0.01$, E-E, $P < 0.01$;
 F-F, $P < 0.01$.

 A-G, $P > 0.05$; B-H, $P > 0.05$; C-I, $P > 0.05$; D-J, $P < 0.01$; E-K, $P < 0.01$;
 F-L, $P < 0.01$.

cinoma and found significant reactivity in 17 out of 22 patients with carcinoma of the lung. No titration experiments were performed, and the antigenic activity reported seemed to vary with the protein concentration of the extracts. The concentration range was from 227–900 $\mu\text{g/ml}$.

Tissue extract concentrations below 10 $\mu\text{g protein/ml}$ were not employed in the present study. However, some migrations were set up in the LMAT using 1000 μg of tissue extract/ml in both controls and renal carcinoma patients. No significant toxic inhibition of leucocytes could be detected.

At present the reason for the non-reactivity in LMAT is not clear. One possibility is as follows: The study of Boddie et al. (7) using KCl-extracts of tumours seem to indicate that the degree of solubilization of the antigen may be critical. With crude homogenates like those employed in the present study a proportion of the relevant tumour antigen may not be in the solubilized state that may be of importance in the LMAT. If so, it will be necessary to postulate a basic difference in the biology of LMAT and LMCT since crude antigens induce significant leucocyte inhibition in the latter.

Efforts in our laboratory are presently directed towards solubilization of the antigens reactive in LMCT and towards an indirect two-step MIF assay to elucidate the mechanisms of leucocyte function in the two techniques.

SUMMARY

A comparison was made between the direct capillary tube leucocyte migration technique (LMCT) and the direct agarose

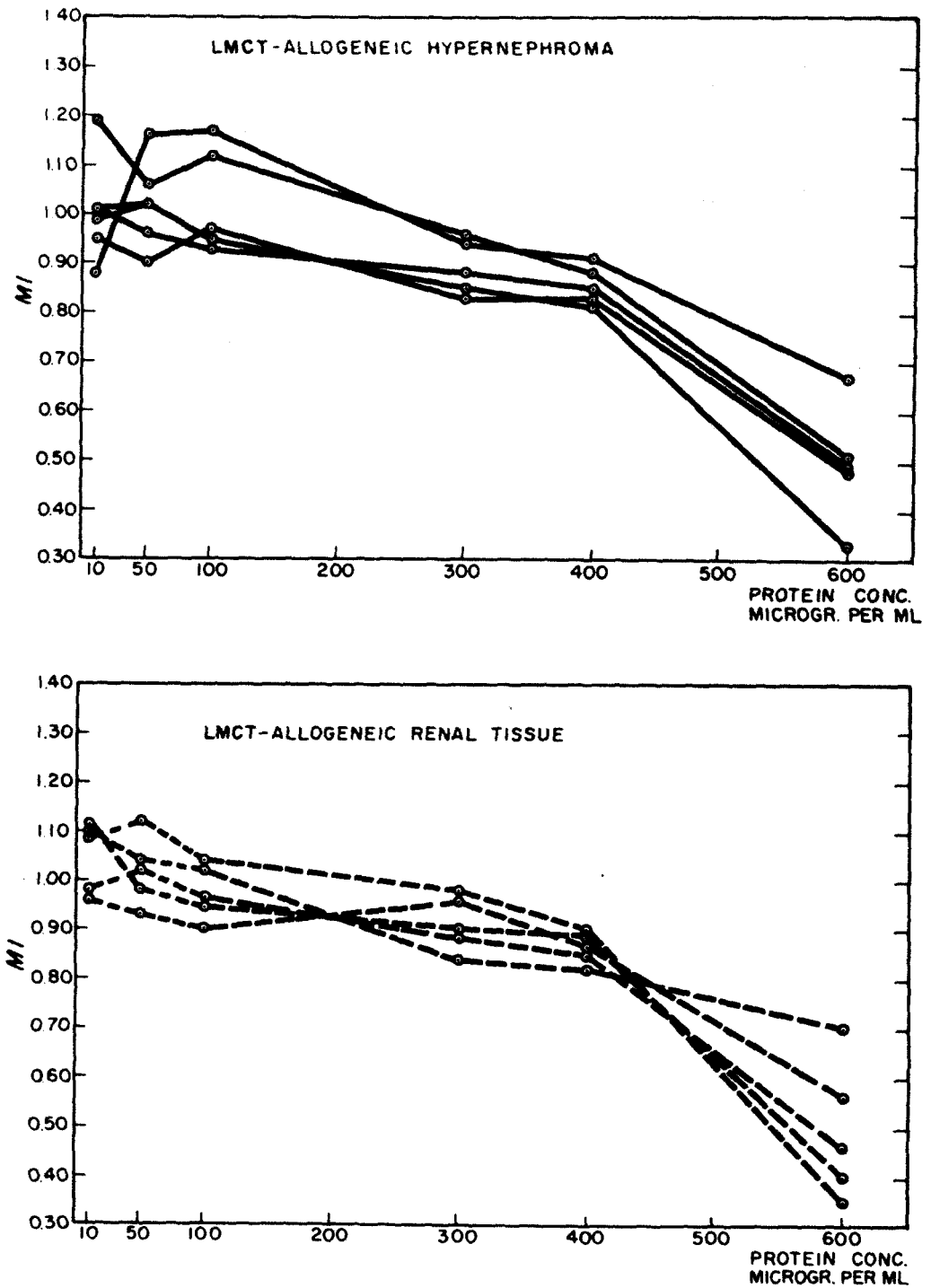


Fig. 1.

Titration curves in LMCT from blood donors using allogeneic hypernephroma and corresponding normal renal tissue extracts.

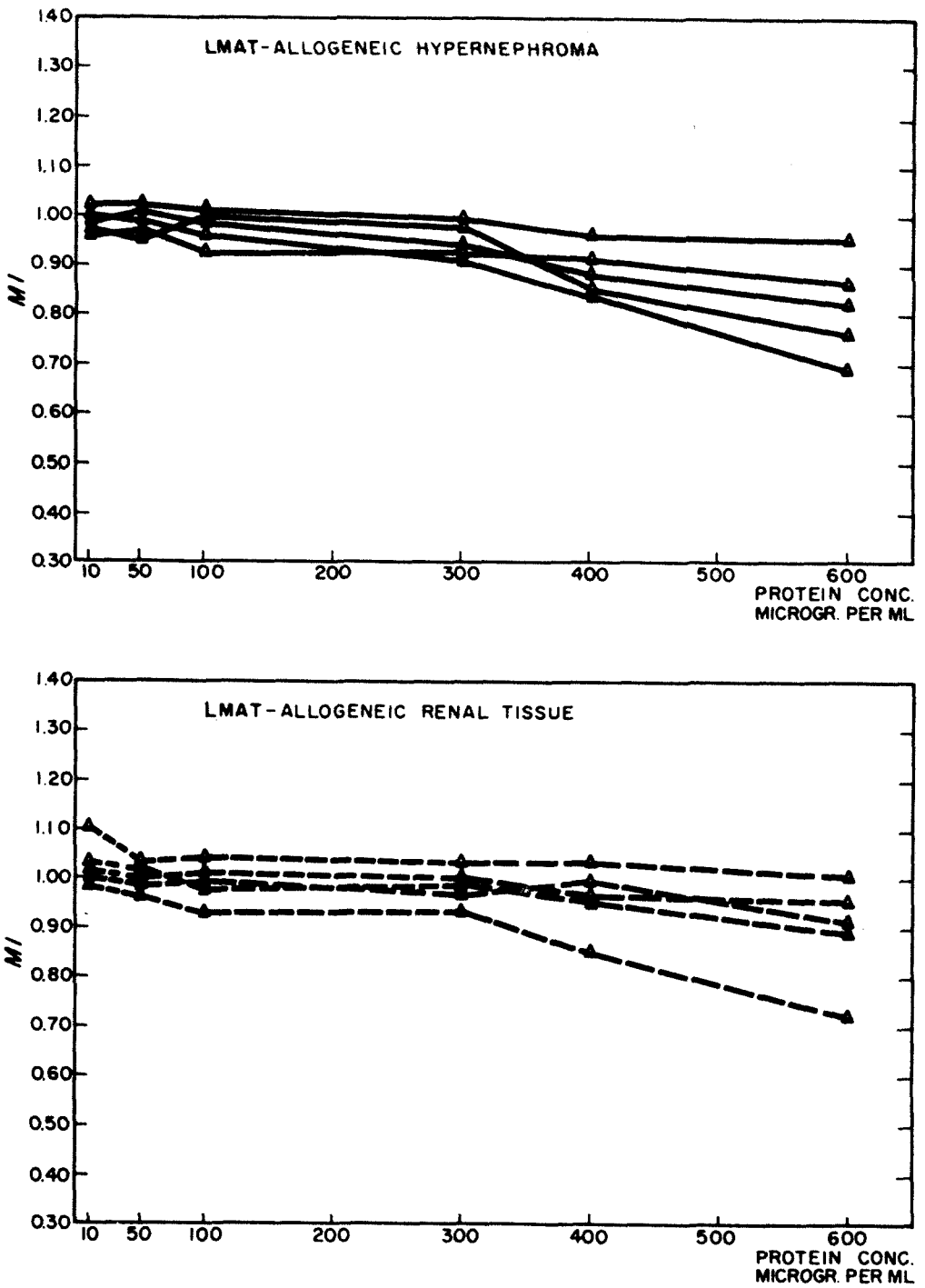


Fig. 2.

Titration curves in LMAT from blood donors using allogeneic hypernephroma and corresponding normal renal tissue extracts.

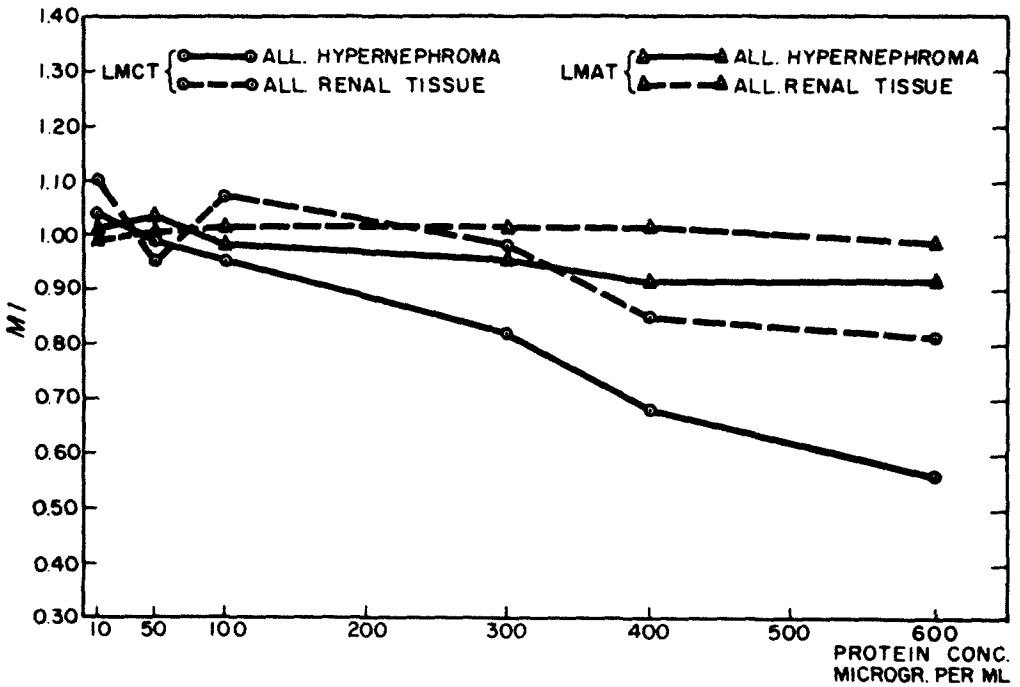
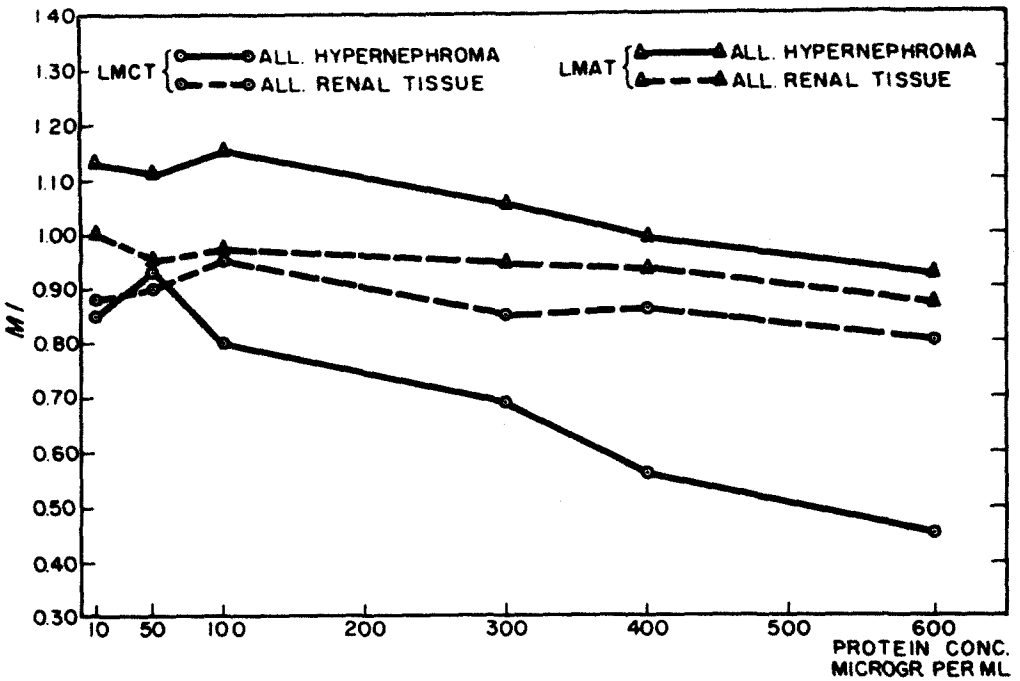


Fig. 3.

Individual titration curves from patients no. 1 and no. 2 (Table 1) in LMCT and LMAT.

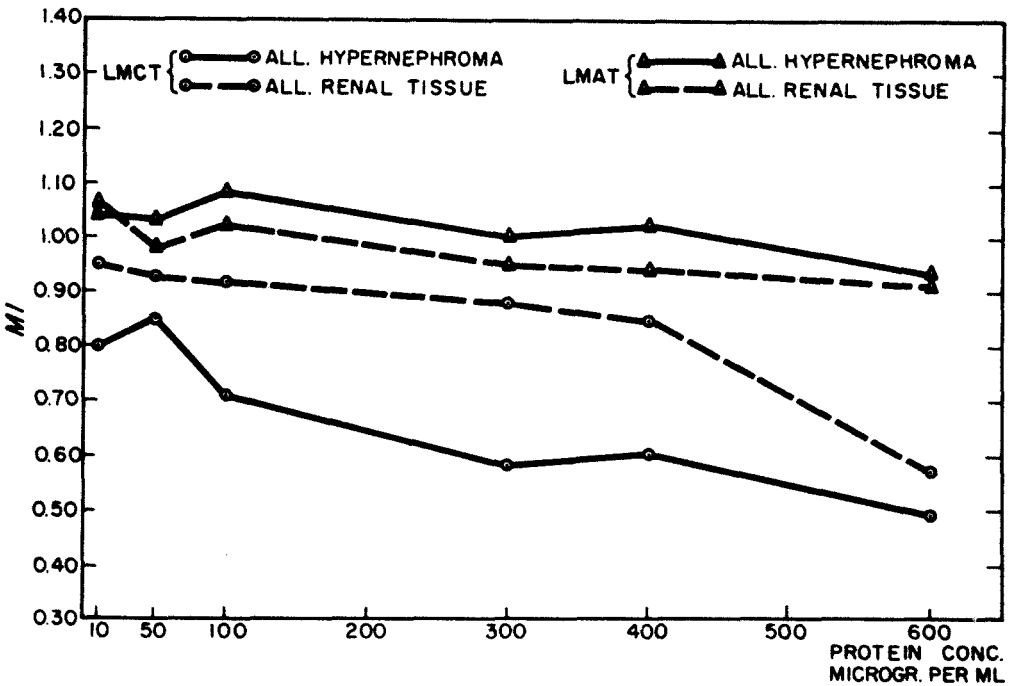
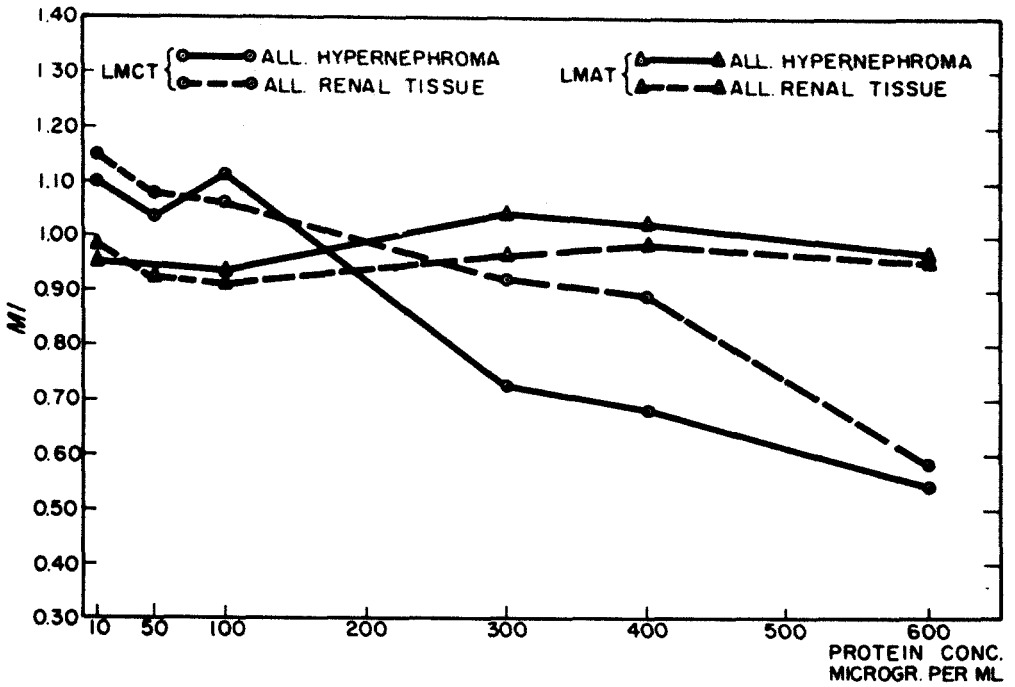


Fig. 4.

Individual titration curves from patients no. 3 and no. 4 (Table 1) in LMCT and LMAT.

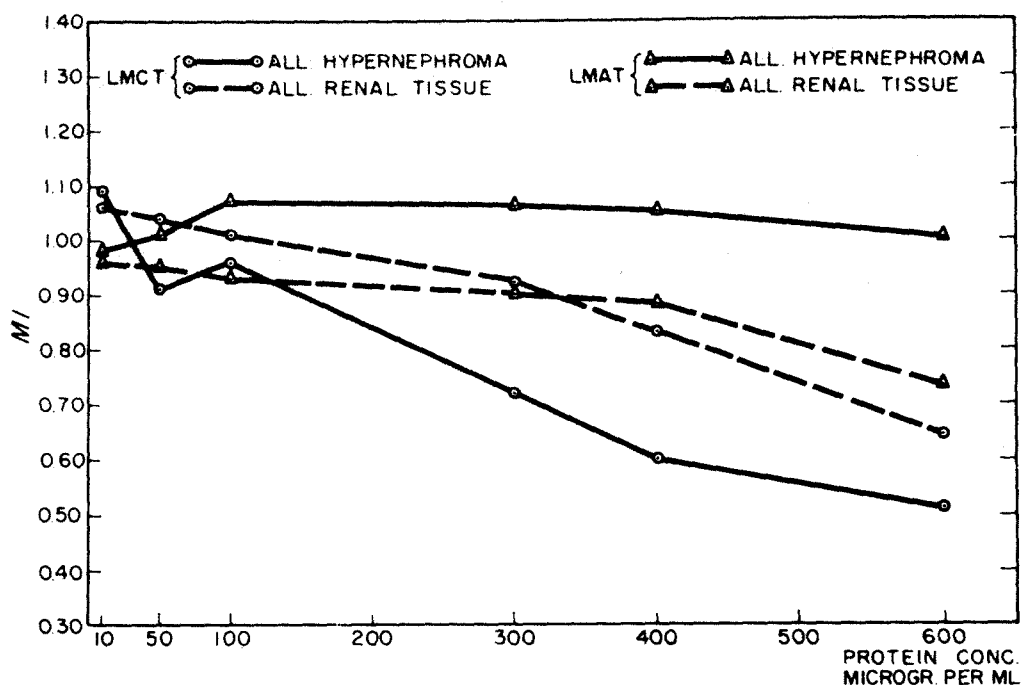


Fig. 5.

Individual titration curves from patient no. 5 (Table 1) in LMCT and LMAT.

leucocyte migration technique (LMAT) using crude homogenates of tumour tissue as antigen in patients with renal carcinoma. A state of specifically altered reactivity of leucocytes from patients with renal carcinoma as compared with normal control persons towards allogeneic hypernephroma extracts could be detected in the LMCT but not in the LMAT. The reactivity in the LMCT was manifest at protein concentrations of 300 and 400 $\mu\text{g}/\text{ml}$. The study indicates that in contrast to the LMCT, the LMAT cannot be used for detection of TCMH towards allogeneic hypernephroma tissue extracts in patients with renal carcinoma.

ACKNOWLEDGEMENTS

This investigation was supported by the Danish Cancer Society. The authors want to thank Mrs Anne Kjerulff, and Mrs Jette Hassing for competent technical assistance.

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