

From the Laboratory for Clinical Immunology, Medical Department TA and Surgical Department C, Rigshospitalet, Copenhagen, Denmark.

TUMOUR-DIRECTED, CELL-MEDIATED HYPERSENSITIVITY IN MAN

*A Critical Review with Special Reference to
Leucocyte Migration Assays*

By

MOGENS KJAER

The introduction of *in vitro* techniques for the detection of tumour-directed, cell-mediated hypersensitivity (TCMH) in man in the late sixties (6, 77, 137) has led to a considerable escalation of our knowledge of the relationship between human solid malignant tumours and the immune response. Three basic types of techniques have been employed: 1) Cytotoxicity assays, 2) lymphocyte transformation techniques, and 3) leucocyte migration techniques (LMT). Several reviews have discussed both technical and clinical problems especially in the cytotoxicity systems (6, 40, 74, 77, 79, 100, 110, 118, 122, 137, 140). Concerning LMT, only scattered information exists as to its application in human tumour immunology.

It is the purpose of the present review to give a brief survey of the various *in vitro* methods currently employed for detection of TCMH in man, and especially to discuss recent technical modifications of the LMT and the results obtained with these techniques in human tumour immunology. Moreover, it is intended to give a survey of LMT-studies in human renal carcinoma (88-97), and on this background to present a general hypothesis concerning the interrelations between TCMH and renal carcinoma in different clinical stages.

TABLE 1

In vitro Techniques for Demonstration of TCMH in Man.

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- I. *Techniques in which whole intact tumour cells are mainly used as antigenic preparation.*
- A. Cytotoxicity techniques.
1. Colony inhibition.
 2. Dilute agar colony inhibition.
 3. Microcytotoxicity.
 4. Inhibition of incorporation of thymidine-³H.
- Isotope release techniques.
1. ⁵¹Chromium release.
 2. Thymidine-³H and ¹²⁵I-iododeoxyuridine release.
 3. ³H-proline release.
- B. Lymphocyte transformation, using Mitomycin-C treated tumour cells.
- II. *Techniques in which soluble tumour extracts are mainly used as antigenic preparation.*
1. Lymphocyte transformation.
 2. Leucocyte adherence inhibition.
 3. Cell migration inhibition.
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In vitro Techniques for Demonstration of TCMH in Man

As shown in Table 1 two principal categories of techniques exist for *in vitro* study of TCMH in man:

- I. Techniques in which intact tumour cells are mainly used as antigenic preparation.
- II. Techniques in which soluble extracts of tumour material are most commonly used as antigenic preparation.

The colony inhibition (CI) technique was first developed by Hellström in 1967 (68). This method measures the ability of sensitized lymphocytes to reduce the cloning efficiency of tumour cells cultured in plastic petri dishes. The effect is determined by visual counting of tumour cell colonies. This assay was used by Hellström et al. in a number of investigations on TCMH in several types of malignant tumours (69, 70, 71, 73). To a great extent these studies formed the basic concept of TCMH in man as it is known to-day.

In 1971 Heppner & Kopp introduced a dilute agar CI test (41, 75, 76) and presented data very similar to those obtained with the Hellström CI test. One of the advantages of this test system was that it gave the culture of tumour cells a selective advantage over normal cells and also allowed growth of cell types which are unable to adhere to and grow on glass or plastic surfaces. However, the test is rather tedious and time-consuming. CI techniques have never gained wide-spread use outside the groups which originally introduced them, probably due to technical difficulties and the elaborate controls necessary if valid results are to be obtained.

Because of these limitations Takasugi & Klein introduced a microcytotoxicity test using survival of single cells carried out in microtest plates (147). A considerable number of studies have been performed with this assay (25, 26, 121, 126, 148, 154) which is probably the test system most commonly used in studies of TCMH in man. Other modifications of the microcytotoxicity test have been employed (71, 72, 125). Recently, some laboratories have obtained results indicating specificity problems with microcytotoxicity assays (78, 82, 148). These difficulties will not be discussed here. Several critical papers have been published (10, 80). Cytotoxicity tests require at the end a visual cell counting. Methods have been introduced, however, in which evaluation of the cytotoxicity is automatized by the release of radioactive isotopes from target cells on contact with specifically sensitized lymphocytes. The $^{51}\text{Chromium}$ release technique originally described by Brunner et al. (24) has been used in several studies of TCMH (81, 124, 139), and the thymidine- ^3H and ^{125}I -iododeoxyuridine assays of Jagarlamoody et al. (86) and Cohen et al. (39) likewise have been employed. Finally, the inhibition of incorporation of thymidine- ^3H (119, 120), and the ^3H -proline technique of Bean et al. (12) have been used in a few studies (12, 116, 119, 120).

Lymphocyte transformation techniques based on the uptake of ^{14}C -thymidine by antigen-stimulated lymphocytes have especially been employed by Stjernswärd et al. (141–143, 155) in

studies of various solid tumours. In these studies Mitomycin-C treated, intact tumour cells were used as stimulating agent. Recently Vanky et al. (156) and others (43, 134) have used lymphocyte transformation with 3 M KCl-extracts of tumour material. In most studies of lymphocyte transformation with tumour extracts, control studies with corresponding normal tissues have not been done, thus leaving considerable doubt as to the specificity of the reactions observed.

In 1972 Halliday & Miller (65) introduced the leucocyte adherence inhibition (LAI) technique for the study of TCMH. This technique is based on the observation that a proportion of leucocytes adhere to glass haemocytometer surfaces. When incubated with tumour extracts the number of adherent cells will be reduced if they are sensitized, as compared to a non-sensitized cell population. The technique is rapid. An answer can be available in a few hours. The LAI technique was subsequently employed in several studies of TCMH in human solid tumours (18, 62, 66, 107, 108, 127). Also modifications in test tubes (62), and an indirect LAI technique for testing of supernatants have been introduced (108). They have not yet been extensively employed.

Selection of Technique for Clinical Investigation

For studies of TCMH in human solid tumours it must be considered essential to detect TCMH towards individual tumours in both autologous and allogeneic situations and to evaluate, if possible, the interrelationship of TCMH and tumour antigenicity. This rules out the use of assays employing established tumour cell lines in culture. For specificity reasons it is of importance to employ comparable cells from corresponding normal (not tumour infiltrated) tissue. Microcytoxicity assays normally employ fibroblast cell lines as control, which cannot be considered satisfactory (79). For these reasons a technique employing soluble tissue extracts as antigen is necessary. Concerning lymphocyte transformation techniques it has been shown by Dean et al. (43) in breast cancer that

allogenic tumour extracts produce stimulation of normal lymphocytes. Thus, it is unlikely that allogenic extracts can be routinely used in situations not allowing autologous testing (preoperatively) in lymphocyte transformation techniques.

Two types of techniques remain:

- a) The LAI technique of Halliday et al. (65).
- b) The LMT of Bendixen & Søbørg (13, 144).

Both techniques employ soluble antigens, have so far shown acceptable specificity, and are rapidly performed. However, the value of the LAI technique as a parameter of cell-mediated hypersensitivity (CMH) in man has not yet been established for instance by correlating the results with intracutaneous reactivity. For these reasons the LMT was chosen for further consideration, and will be discussed in detail below.

Cell Migration Inhibition Techniques

On contact with specific antigens *in vitro* sensitized human lymphocytes release into the medium a number of soluble factors with a variety of biological properties. Some of these factors, lymphokines, are mediators in CMH. The biology of lymphokines has been the subject of several extensive reviews (115, 157). One of these factors, migration inhibition factor (MIF), has the property to inhibit the migration of cell populations *in vitro* on an appropriate surface. This biological activity has formed the basis of a number of *in vitro* techniques for the study of CMH in man. As shown in Table 2, two principal categories of *in vitro* techniques for cell migration inhibition exists:

- I. Direct techniques, in which MIF is released by and exerts its activity on the same migrating cell population.
- II. Indirect techniques, in which sensitized lymphocytes in a first step are stimulated to release MIF on contact with antigen. In a second step the biological activity of the cell-free supernatant is registered through its effect in an independent (indicator) migrating cell population.

Søbørg & Bendixen first adapted direct migration inhibition

TABLE 2

Principal Categories of Cell Migration Inhibition Techniques for the Study of Cell-Mediated Hypersensitivity in Man.

I. *Direct techniques.*

A. Leucocyte migration capillary tube technique (LMCT).

Migrating cells:

1. Leucocytes from peripheral blood.
2. Mononuclear cells from peripheral blood.
3. *In vitro* cultured lymph node cells.

B. Leucocyte migration agarose technique (LMAT).

II. *Indirect techniques.*

A. LMCT.

Migrating cells:

1. Leucocytes from peripheral blood.
2. *In vitro* cultured lymphocytes.
3. Guinea pig macrophages.

B. LMAT.

Migrating cells:

1. Leucocytes from peripheral blood.
 2. Granulocytes from peripheral blood.
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to human peripheral leucocytes migrating from capillary tubes in siliconized glass chambers (LMCT) (13, 144-146). The technique was later employed by other investigators (53, 131, 132). The results of the assay were shown to correlate well with intracutaneous reactivity to purified protein derivative (PPD) (131), and a number of other antigens (53, 146), and has since then been used for studying CMH in a multitude of clinical conditions. The results have been extensively reviewed (14, 130).

Several modifications using other types of migration chambers, and other types of migrating cell populations have been developed since then (38, 50-52, 55, 106, 112, 149, 151, 152), and have been used with a variety of antigens.

There are two principal ways of administering antigen in the test. In the original modification the antigen is present in

the culture chamber during the total culture period (13, 144-146). Other investigators (38, 112) use pre-incubation of cells with antigen followed by direct migration of the pre-incubated cells in antigen-free medium.

Because of the need for less cell- and antigen-consuming techniques Federlin et al. (55, 106) introduced a micro-modification using 0.5 ml disposable polystyrene migration chambers and microcapillaries containing only 4×10^5 cells. This technique was first standardized with PPD as antigen, but has later been used with a number of other antigens (8, 90-92, 111, 112).

In 1971 Clausen introduced a modification in which pre-incubated peripheral leucocytes migrated under solid agar over the bottom of plastic petri dishes (31). With this leucocyte migration agarose technique (LMAT) Clausen (31, 34) and later Astor et al. (9) examined a variety of technical parameters mainly using PPD, but also streptokinase-streptodornase, candida, and cocci were used. The response in the LMAT was shown to correlate well with intracutaneous reactivity. The technique is saving in cells and antigen as compared to LMCT (15, 17) and has been increasingly employed.

Supernatants from antigen-exposed lymphocyte cultures have been investigated both in LMCT and LMAT using a variety of cell populations as migrating cells (Table 2). In the LMCT leucocytes from peripheral blood of non-sensitized persons (123) and *in vitro* cultured lymphocytes (57, 58, 150, 153) have been used. The most commonly employed indirect technique, however, has been the one introduced by Thor (153), and by Rockin et al. (128, 129) in which the activity of supernatants is tested on a migrating cell population consisting of guinea pig macrophages. This type of test system has been used in a variety of disease states in man (for review see 130).

In 1972 and 1973 Clausen (32, 33) introduced an indirect technique (two-step-MIF-assay) in which the biological activity of only a few hundred microliters of supernatant was

TABL
*Leucocyte Migration Studies of TCMH again.
 Technical Modification*

Ref.	Technique	Obtained from	Immunoreactive cells		No. of cell washings	No. of cells/lymphocytes per test
			Cell types	Per cent lymphocytes		
4	direct LMCT	blood	leucocytes	app. 40	4	$6-8 \times 10^6$
5	direct LMCT	blood	leucocytes	app. 40	4	$6-8 \times 10^6$
59	direct LMCT	blood	leucocytes	n.i.	2	n.i.
135	direct LMCT	blood	leucocytes	n.i.	3	n.i.
54	direct LMCT	blood	leucocytes	n.i.	5	n.i.
19	direct LMCT	blood	leucocytes	n.i.	4	n.i.
20	direct LMCT	blood	leucocytes	n.i.	4	n.i.
21	direct LMCT	blood	leucocytes	n.i.	4	n.i.
89	direct LMCT	blood	leucocytes	app. 40	3	$6-8 \times 10^6$
88	direct LMCT	blood	leucocytes	app. 40	3	$6-8 \times 10^6$
85	direct LMCT	blood	leucocytes	n.i.	2	n.i.
48	direct LMCT	1) blood	1) leucocytes	n.i.	2	n.i.
		2) lymph nodes	2) lymphocytes	n.i.	2	n.i.
63	direct LMCT micromod.	blood	leucocytes	app. 40	4	n.i.

3 A.

*Solid Tumours in Man: Selected References.**Employed.*

Type	Antigen preparation			Antigen prepared from normal tissues	Estimation of tumour antigenicity	Cut-off level of significant leucocyte migration inhibition
	Dose-response performed	Protein conc. tested	Protein conc. used			
soluble extract Hanks'	+	50-100 150-200 μg/ml	100-150 μg/ml	+	n.s.	MI < 0.80
soluble extract Hanks'	+	50-100 150-200 μg/ml	100-150 μg/ml	+	n.s.	MI < 0.78 MI > 1.23
soluble extract NaCl	n.i.	—	app. 300 μg/ml	+	n.s.	MI < 0.77 MI > 1.57
soluble extract NaCl + ultrasound	n.i.	—	100 μg/ml	+	n.s.	MI < 0.75 MI > 1.20
soluble extract	+	100-10,000 μg/ml	10, 100, 1000 μg/ml	n.s.	n.s.	MI < 0.90
cryostat sections	—	—	—	+	+	MI < 0.75
cryostat sections	—	—	—	+	+	MI < 0.75
cryostat sections	—	—	—	n.i.	+	MI < 0.75
soluble extract Hanks'	+	50, 100, 200, 300, 400, 600 μg/ml	400 μg/ml	+	n.s.	MI < 0.79 MI > 1.19
soluble extract Hanks'	+	50, 100, 200, 300, 400, 600 μg/ml	400 μg/ml	+	n.s.	MI < 0.79 MI > 1.19
soluble extract Hanks'	n.i.	—	100, 300 μg/ml	+	n.s.	MI < 0.58 MI > 0.98
soluble extract PBS + sonication	n.i.	—	100 μg/ml	n.s.	n.s.	MI < 0.80
soluble extract perchloric acid	+	?-150 μg/ml	50 μg/ml	+	n.s.	MI < 0.79 MI > 1.25

TABLE 3

Ref.	Technique	Obtained from	Immunoreactive cells		No. of cell washings	No. of cells lymphocyte per test
			Cell types	Per cent lymphocytes		
47	direct LMCT micromod.	blood	leucocytes	n.i.	3	$1-3 \times 10^6$
87	direct LMCT micromod.	blood	leucocytes	n.i.	3	6×10^5
3	direct LMCT micromod.	blood	leucocytes	n.i.	3	n.i.
95	direct LMCT micromod.	blood	leucocytes	app. 40	3 6 10	$2-3 \times 10^6$
96	direct LMCT micromod.	blood	leucocytes	app. 40	3	$2-3 \times 10^6$
158	direct LMCT micromod. Pasteur pip.	blood	leucocytes	app. 60	3	n.i.
159	direct LMCT micromod. Pasteur pip.	blood	1) leucocytes 2) lymphocytes	app. 60 94-97	3	n.i. n.i.
160	direct LMCT micromod. Pasteur pip.	blood	leucocytes	app. 60	3	n.i.
35	direct LMCT preincubation	blood	leucocytes	n.i.	3	n.i.
105	direct LMCT preincubation	blood	leucocytes	n.i.	3	n.i.
36	direct LMCT preincubation	blood	leucocytes	n.i.	3	n.i.
37	direct LMCT preincubation	blood	leucocytes	n.i.	3	n.i.

(continued).

Type	Antigen preparation			Antigen prepared from normal tissues	Estimation of tumour antigenicity	Cut-off level of significant leucocyte migration inhibition
	Dose-response performed	Protein conc. tested	Protein conc. used			
1) sol. ext. Hanks' 2) fresh + 3) dead tumour cells	n.i.	—	1) 100 µg/ml 2 + 3) 0.5-5 × 10 ⁶ cells/test	+	n.s.	MI < 0.80
soluble extract Hanks'	+	50, 100, 150, 200 µg/ml	50, 100, 150, 200 µg/ml	+	n.s.	MI < 0.80
soluble extract NaCl	n.i.	—	300, 500 µg/ml	Grave's disease	n.s.	n.i.
soluble extract Hanks'	+	10, 50, 100, 300, 400, 600 µg/ml	10, 50, 100, 300, 400, 600 µg/ml	+	+	MI < 0.79 MI > 1.19
soluble extract Hanks'	+	10, 50, 300, 400, 600 µg/ml	10, 50, 100, 300, 400, 600 µg/ml	+	+	MI < 0.79 MI > 1.19
1) sol. ext. Eagle 2) + centrifug. 3) ultrasound	n.i.	—	n.i.	+	n.s.	n.i.
1) sol. ext. Eagle 2) + centrifug. 3) ultrasound	n.i.	—	n.i.	+	n.s.	n.i.
soluble extract Eagle + ultrasound	n.i.	—	1-5 mg/ml	+	n.s.	n.i.
soluble extract Eagle	+	12.5-200 µg/ml	12.5-100 µg/ml	+	n.s.	n.i.
soluble extract Eagle	+	12.5-200 µg/ml	12.5-100 µg/ml	n.s.	n.s.	n.i.
soluble extract Eagle	+	12.5-200 µg/ml	12.5-100 µg/ml	n.s.	n.s.	n.i.
soluble extract Eagle	+	12.5-100 µg/ml	12.5-100 µg/ml	+	n.s.	n.i.

TABLE 3

Ref.	Technique	Obtained from	Immunoreactive cells		No. of cell washings	No. of cells: lymphocyte per test
			Cell types	Per cent lymphocytes		
38	direct LMCT preincubation	blood	leucocytes	n.i.	2	n.i.
133	direct LMCT preincubation	blood	leucocytes	n.i.	3	n.i.
111	direct LMCT micromod. preincubation	blood	leucocytes	35-45	2	n.i.
112	direct LMCT micromod. Pasteur pip.	blood	leucocytes	n.i.	2	n.i.
109	direct LCMT micromod. Pasteur pip.	blood	lympho-monocytes	app. 80	2	n.i.
28	direct LMCT preincubation	blood	lympho-monocytes	n.i.	3	n.i.
104	direct LMCT preincubation	blood	lympho-monocytes	n.i.	3	n.i.
56	direct LMCT preincubation	blood	lympho-monocytes	n.i.	n.i.	n.i.
8	direct LMCT micromod.	blood	1) leucocytes	1) n.i.	n.i.	n.i.
			2) mononuclear cells	2) 94-96	n.i.	n.i.
			3) granulocytes	3) 3-5	n.i.	n.i.
22	direct LMAT	blood	leucocytes	n.i.	4	n.i.
94	direct LMCT direct LMAT	blood	leucocytes	app. 40	6	1) 1.5×10^6
					6	2) 3×10^6
123	indirect LMCT	blood	lympho-monocytes	app. 98	2	5×10^6

(continued).

Type	Antigen preparation			Antigen prepared from normal tissues	Estimation of tumour antigenicity	Cut-off level of significant leucocyte migration inhibition
	Dose-response performed	Protein conc. tested	Protein conc. used			
soluble extract Eagle	+	12.5-100 $\mu\text{g/ml}$	12.5-100 $\mu\text{g/ml}$	+	n.s.	n.i.
formalin-fixed tumour cells	+	leuco/tumour cells: 100:1-1000:1	leuco/tumour cells: 100:1, 200:1	+	n.s.	n.i.
soluble extract 3M KCl	+	?-1000 $\mu\text{g/ml}$	500-750 $\mu\text{g/ml}$	+	n.s.	MI < 0.80
soluble extract 3M KCl	+	?-1500 $\mu\text{g/ml}$	500-750 $\mu\text{g/ml}$	+	+	MI < 0.80
soluble extract 3M KCl	+	20-1000 $\mu\text{g/ml}$	20-1000 $\mu\text{g/ml}$	n.s.	n.s.	MI < 0.80
soluble extract NaCl	n.s.	—	3.3 $\mu\text{g/ml}$	n.s.	n.s.	MI < 0.79 MI > 1.16
soluble extract NaCl	n.s.	—	3.3 $\mu\text{g/ml}$	n.s.	n.s.	MI < 0.80
soluble extract PBS	+	n.i.	2,000 $\mu\text{g/ml}$	+	n.s.	MI < 0.80
soluble extract perchloric acid	n.s.	—	10 $\mu\text{g/ml}$	n.s.	n.s.	MI < 0.87 MI > 1.12
soluble extract 3M KCl	+	227-900 $\mu\text{g/ml}$	227-900 $\mu\text{g/ml}$	+	n.s.	MI < 0.70 MI > 1.30
soluble extract Hanks'	+	10, 50, 100, 300, 400, 600, 1000 $\mu\text{g/ml}$	10, 50, 100, 300, 400, 600 $\mu\text{g/ml}$	+	+	MI < 0.79 MI > 1.19
soluble extract NaCl + ultrasound	n.s.	—	1,200 μg	+	n.s.	MI < 0.72

TABLE
*Leucocyte Migration Studies of TCMH against
 Results*

Ref.	Type of tumour	Patients			Grading of TCMH
		Reaction with autologous tumour. No. pos./No. tested	Reaction with allogeneic tumour of the same histological type. No. pos./No. tested	Reaction with corresponding normal tissues. No. pos./No. tested	
4	mammary carcinoma	5/6	n.s.	0/8	n.s.
5	mammary carcinoma	8/22	n.s.	0/22	n.s.
59	squamous cell carcinoma of the cervix ut.	11/14	n.s.	0/1	n.s.
135	various solid tumours	23/40	6/21	0/26	n.s.
54	stomach, colonic cancer, malignant melanoma	n.s.	14/14 11/11 24/31	n.s.	n.s.
19	mammary carcinoma	42/116*)	97/293*)	13/75*)	n.s.
20	mammary carcinoma	36/124*)	152/543*)	5/116*)	n.s.
21	mammary carcinoma	55/189*)	78/328*)	n.i.	n.s.
89	renal carcinoma	8/11	13/18	2/18	n.s.
88	renal carcinoma	4/4	3/4	0/8	n.s.
85	colonic carcinoma	15/31*)	n.s.	0/31*)	n.s.
48	mammary carcinoma	n.s.	1) 7/24 2) 17/24	n.s.	n.s.
63	colonic carcinoma	n.s.	15/22	5/21	n.s.
47	various solid tumours	19/35	n.s.	2/12	n.s.
87	mammary carcinoma	31/105	12/64	2/19	n.s.

3 B.

*Solid Tumours in Man: Selected References.
Obtained.*

Correlation with clinical stage	Patients		Types of controls investigated	Controls	
	Effect of serum	Follow-up performed		Tumour	Normal tissue
n.s.	n.s.	n.s.	healthy donors	0/8	0/8
n.f.	n.s.	+	1) healthy donors	0/22	0/21
			2) fibrocystic disease	n.s.	0/9
n.s.	n.s.	n.s.	patients with benign disease	n.s.	0/7
n.f.	n.s.	n.s.	1) healthy donors	n.i.	n.i.
			2) non-related carcinoma	n.i.	n.i.
n.f.	+	n.s.	1) healthy donors	0/20	n.s.
			2) non-related carcinoma	1/11	n.s.
+	n.s.	n.s.	healthy donors	3-7 %	3-7 %
+	n.s.	n.s.	1) healthy donors	14/198*)	3/41*)
			2) benign breast		
+	n.s.	n.s.	1) healthy donors	0/85*)	n.i.
			2) benign breast	1/91*)	n.i.
+	n.s.	n.s.	1) healthy donors	3/45	4/45
			2) ben. ren. dis.	0/6	0/6
			3) non-rel. carc.	0/17	0/17
n.s.	+	n.s.	healthy donors	0/8	0/8
n.f.	n.s.	n.s.	healthy donors	0/84	0/84
n.f.	n.s.	n.s.	open heart surgery	1) 0/10	1) n.s.
			patients	2) 0/10	2) n.s.
n.f.	+	n.s.	healthy donors	0/15	1/15
+	n.s.	+	n.s.	—	—
n.f.	n.s.	+	1) healthy donors	5/104	1/15
			2) non-rel. carcinoma		
			3) ben. breast disease		
			4) pregnant women		

TABLE 3 B.

Ref.	Type of tumour	Patients			Grading of TCMH
		Reaction with autologous tumour. No. pos./No. tested	Reaction with allogeneic tumour of the same histological type. No. pos./No. tested	Reaction with corresponding normal tissues. No. pos./No. tested	
3	thyroid carcinoma	0/2	0/7	n.i.	n.s.
95	renal-carcinoma	n.s.	4/16 14/16 9/9	0/16 0/16 0/9	+
96	renal carcinoma	7/12	23/49	3 %	+
158	various types of solid carcinoma	14/16	n.i.	n.i.	n.s.
159	various types of solid carcinoma	1) 19/21 2) 2/2	n.i.	n.i.	n.s.
160	various types of solid carcinoma	13/41	n.i.	n.i.	n.s.
35	1) malignant melanoma 2) mammary carcinoma	4/7 6/8	37/46 28/45	n.i. 1/11	n.s. n.s.
105	malignant melanoma	n.s.	10/10	n.s.	n.s.
36	malignant melanoma	8/10	33/55	n.s.	n.s.
37	1) malignant melanoma 2) mammary carcinoma	1) 8/10 2) 11/14	1) 33/55 2) 38/73	1) n.s. 2) 5/26	n.s. n.s.
38	mammary carcinoma	19/32	78/106	1/5	n.s.
33	1) malignant melanoma 2) mammary carcinoma	1) 6/10 2) n.s.	1) 38/69 2) 55/81	normal liver 1) 2/10 2) 0/3	n.s. n.s.

(continued).

Correlation with clinical stage	Patients		Types of controls investigated	Controls	
	Effect of serum	Follow-up performed		Tumour	Normal tissue
n.s.	n.s.	n.s.	healthy donors	n.i.	n.i.
+	n.s.	n.s.	1) healthy donors	0/44	0/41
			2) ben. renal disease	0/33	0/38
			3) non-rel. carcinoma	0/18	0/18
+	n.s.	+	1) healthy donors	3 %	6 %
			2) ben. renal disease	5 %	3 %
			3) non-rel. carcinoma	1 %	3 %
n.s.	n.s.	n.s.	n.i.	—	—
n.s.	n.s.	n.s.	n.i.	—	—
n.s.	n.s.	+	n.i.	—	—
n.s.	n.s.	n.s.	healthy donors	6/31	n.s.
n.s.	n.s.	n.s.		18/64	n.s.
n.s.	n.s.	n.s.	n.s.	—	—
+	n.s.	n.s.	healthy donors	9/63	n.s.
+	n.s.	n.s.	1) healthy donors + various diseases	1) 9/63	1) n.s.
+			2) healthy donors + various diseases	2) 19/99	2) 1/12
+	n.s.	+	1) healthy donors	1) 8/26	1-9 %
			2) non-rel. carcinoma	2) 2/28	
			3) various diseases	3) 1/7	
			4) ben. breast disease	4) 36/193	
n.s.			1) healthy donors + various diseases	1) 4/31	1) n.s.
n.s.	n.s.	n.s.	2) healthy donors + various diseases	2) 16/46	2) n.s.

TABLE 3 B.

Ref.	Type of tumour	Patients			Grading of TCMH
		Reaction with autologous tumour. No. pos./No. tested	Reaction with allogeneic tumour of the same histological type. No. pos./No. tested	Reaction with corresponding normal tissues. No. pos./No. tested	
111	mammary carcinoma	1/2	20/26	0/9	n.s.
112	malignant melanoma	n.i.	48/79	benign naevus 0/13	n.s.
109	colonic carcinoma	1/1	n.s.	n.s.	n.s.
28	colonic carcinoma	n.s.	24/27	n.s.	n.s.
104	colonic carcinoma	n.s.	12/12	n.s.	n.s.
56	medullary carcinoma of the thyroid	n.s.	10/11	3/11	n.s.
8	colonic carcinoma	n.s.	1) 5/9 2) 7/7 3) 0/9	n.s.	n.s.
22	pulmonary carcinoma	3/3	17/22	1/22	n.s.
94	renal carcinoma	n.s.	1) 5/5 2) 0/5	1) 0/5 2) 0/5	+
123	renal carcinoma	17/19	6/7	0/18	n.s.

Footnotes concerning Table 3 A and 3 B:

*) indicates number of tests.

n.f. = not found.

n.i. = not indicated.

n.s. = not studied.

+) indicates positive results.

—) indicates lack of information owing to the set-up of the investigation.

(continued).

Correlation with clinical stage	Patients		Types of controls investigated	Controls	
	Effect of serum	Follow-up performed		Tumour	Normal tissue
n.s.	n.s.	n.s.	1) healthy donors 2) ben. breast disease 3) unrel. carcinoma	1) 3/25 2) 2/10 3) 2/9	1) 0/4 2) 0/2 3) n.s.
n.f.	n.s.	n.s.	1) healthy donors 2) unrel. carcinoma	1) 3/50 2) 4/27	1) n.s. 2) n.s.
n.s.	n.s.	n.s.	healthy donors	n.i.	n.i.
n.s.	n.s.	n.s.	1) healthy donors 2) unrel. carcinoma 3) benign g-i. disease	3/52	n.s.
n.f.	n.s.	+	n.i.	0/17	n.s.
n.s.	n.s.	n.s.	1) healthy donors 2) family members 3) spouses	1) 5/13 2) 1/6 3) 2/7	1) 1/10 2) 3/6 3) n.i.
n.s.	+	n.s.	1) healthy donors 2) benign disease	0/6 0/6 0/6	n.s. n.s. n.s.
n.f.	n.s.	n.s.	1) healthy donors 2) benign lung disease 3) non-rel. carcinoma	1) 1/16 2) 0/11 3) 5/25	1) n.s. 2) n.s. 3) n.s.
n.s.	n.s.	n.s.	healthy donors	1) 0/9 2) 0/5	1) 0/9 2) 0/5
n.s.	n.s.	n.s.	1) healthy donors 2) non-rel. carcinoma	1) 0/14 2) 1/9	1) 0/19 2) n.s.

tested in LMAT using peripheral leucocytes from non-sensitized individuals as migrating indicator cells. The use of purified granulocyte preparations as migrating cells was later introduced by Gorski et al. (60). This technique should be suitable for investigating numerous aspects of CMH in man (84).

Leucocyte Migration Studies of TCMH against Solid Tumours in Man

Approximately 50 papers have up to now been published concerning TCMH against human solid tumours studied by means of leucocyte migration techniques. In Table 3 are shown the main features of the technical modifications employed and the most important results obtained with these techniques. In patient materials combined of various tumour types (47, 135, 158-160) data on non-solid tumours have been excluded from the Table. Moreover, some investigations have been published (7, 61, 101) where the LMT has been used to monitor TCMH during immunotherapy. Data from these studies have not been included in the Table. Finally, only studies in which both antigen responsive and indicator cell populations are of human origin have been considered. Studies with guinea pig macrophages as indicator cells (1, 83, 102, 136) have not been included in the Table.

It is apparent that the vast majority of studies have been performed with the direct LMCT in either the original modification by Bendixen & Søborg (13) or in the modification introduced by Maini et al. (106). Some groups use pre-incubation of antigen and cells before collecting the cells in capillary tubes. Practically no data are available on the reactivity of cells from other sources than blood. There is a great need for studies on regional lymph node cells and immunoreactive cells within tumours. The number of cell washings used vary considerably, 2-10 times, most studies using three washings. The importance of this point will be discussed separately. Most frequently, soluble extracts have been used as antigenic preparation. The protein concentrations used vary

widely, between 3.3 and 1.200 μg per ml culture medium. Most studies have employed normal tissue extracts as control, but practically no studies have used systematic evaluation of variation in antigenicity of different tumour antigen preparations. The problems of dose-response, cut-off limits of significant migration inhibition, and variation in tumour antigenicity, are unsatisfactorily illuminated in most studies and will be discussed below.

Concerning the results obtained, cross-reactivity between tumours of the same histological type is a predominant feature. Correlation between significant response and clinical stage has been variably reported, the correlation, if found, most often being reduction in reactivity with advancing tumour burden. The effect of serum on the *in vitro* reactivity (autologous/allogenic serum from patients with carcinoma of the same histological type) has been studied in a few investigations, and it seems established that the technique is well suited for examinations of serum inhibitory effect. Follow-up has been performed in a limited number of studies, the general finding being progressive loss of reactivity weeks to months after radical tumour resection. The protocols of control investigations vary considerably, some authors using extensive control testing of different patient groups, others using practically no control testing at all. For matter of specificity of the response it must be a basic claim that tumour patients as well as control persons are tested with: 1) extracts of tumour tissue, 2) extracts of corresponding normal tissue, and 3) that control persons of various types: patients with benign tumours, non-related carcinoma, non-tumourous disease, if possible involving the same organ system as the tumour type tested, and healthy controls (blood donors) are included in the investigation and tested with the same tissue extracts. Few papers fulfill these requirements.

A review of the literature describing detection of TCMH by means of LMT in patients with solid tumours shows wide variations in the technical modifications employed, especially as to antigen preparation and antigen concentration used.

There is an urgent need for standardization, particularly of the direct LMCT regarding cell preparations, media, cell washings, antigen preparation and concentration, etc.

Earlier Studies of TCMH and General Immunocompetence (GIC) in Human Renal Carcinoma

An immunological defense against human renal carcinoma has been suspected for many years mainly based on case reports of spontaneous regression of established, clinically detectable tumours (11, 49, 64, 99, 193).

In 1970 Stjernswärd et al. (142) showed increased lymphocyte stimulation by autologous tumour cells in 3/6 patients with renal carcinoma. Subsequently, TCMH was demonstrated in 3/3 patients by Hellström et al. (71) using colony inhibition and cytotoxicity tests. TCMH was demonstrated in both autologous and allogeneic combinations. Bubenik et al., who also used the microcytotoxicity test, found TCMH against autologous and allogeneic renal carcinoma cells in 12/19 patients (27), and this finding was supported by Cummings et al. (42) in 8/13 patients and by Elhilali et al. (45). Elhilali et al. also demonstrated blocking activity of serum from patients with renal carcinoma and noted that cell lines from metastatic sites were less antigenic than cell lines derived from the primary tumour. Finally, Pappas & Schwarze (123) using an indirect LCMT with peripheral leucocytes from normal donors as indicator cells showed significant migration inhibition in 17/19 patients with renal carcinoma tested with autologous tumour preparations and in 6/7 tested with allogeneic preparations.

Most studies of GIC in renal carcinoma have been performed on a very limited number of patients included in series with other types of genitourinary carcinoma (2, 29, 30, 44, 113, 114). *In vitro* studies have reported a defective response to phytohaemagglutinin (PHA) in transformation techniques (2, 30, 113, 114) in some studies due to an inhibiting factor in plasma (30, 114). *In vivo* studies have used skin testing

with dinitrochlorobenzene (DNCB) (29), and dinitrofluorobenzene (DNFB) (44) and have shown defective reactivity to these compounds.

Three papers have been published concerning GIC in larger groups of patients with renal carcinoma (23, 46, 67). In two studies Brosman et al. (23) and Hausman et al. (67) comprising 31 and 24 patients, respectively, monocyte function was evaluated by means of monocyte chemotaxis assay (117) and intracutaneous reactivity by response to recall antigens (monilia, streptokinase-streptodornase, PPD) and by reactivity to DNCB. Defective monocyte function was found to be predominant among the patients, but no significant correlation could be established to clinical stage. Stage-related defects were found in the responses to DNCB and recall antigens. In a study of 103 patients with varying stages of urologic cancer, in which 15 were patients with renal carcinoma, Elhilali et al. (46) examined T- and B-cells in peripheral blood, response to PHA, PHA-dependent cellular cytotoxicity, and antibody dependent cellular cytotoxicity against antibody-coated heterologous target cells. Significant reduction in the percentage of both T- and B-cells was found in patients as compared with controls. Also a significant reduction of PHA proliferative responses was evident among the patients. No differences were found between patients and controls using the PHA-dependent or antibody-dependent cytotoxicity assays.

It seems fair to conclude that the demonstration of TCMH in patients with renal carcinoma is well established by a number of *in vitro* techniques in both autologous and allogeneic situations. Little information, however, is available concerning the relations between clinical stage and TCMH and the *in vivo* relevance of the *in vitro* detected phenomena. Concerning GIC there is evidence as to the existence of a decreased response to PHA, DNCB, and recall antigens, and a defective monocyte function. Like in TCMH no conclusive data are available concerning the relation between clinical stage and GIC or, as a point of the utmost importance, the relation between detectable defect of GIC and TCMH.

Studies of TCMH and Blocking Phenomena by Means of the LMCT in Patients with Renal Carcinoma

Such studies were designed with the intention:

1. to develop a simple, rapid, reproducible, and quantitative assay of TCMH on basis of the original LMCT (13, 144-146),
2. with this technique to perform systematic studies on different components of the immune response in a homogeneous patient population, and thereby illustrate:
 - a) the existence of TCMH against autologous and allogeneic renal carcinoma tissue in patients with renal carcinoma,
 - b) the correlations between TCMH and the clinical stage of the disease, and the prognostic significance of TCMH in a prospective clinical investigation,
 - c) factors of importance for the immunological non-reactivity often found in patients with disseminated carcinoma.

Details of these investigations are published elsewhere (16, 88-97). In the present review only the conclusions will be summarized.

LMCT

For investigation of TCMH in human renal carcinoma the direct LMCT in Maini et al.'s modification (106) is preferable because: 1) it is easy to perform, 2) it is much less time-consuming than indirect techniques, 3) there is no need for separate indicator cell populations, and 4) it is cell- and antigen-saving compared with the original LMCT-modification. The choice between the direct LMCT and the direct LMAT was made on basis of a study comparing TCMH in renal carcinoma patients detected by means of both LMCT and LMAT. It was found that specific reactivity in the patients could not be detected in the LMAT (94), presumably because of the relatively crude tissue extract preparations used.

The micromodified direct LMCT and the preparation of

tissue extracts have been described in considerable detail in several publications (16, 90, 92). The variation in results of repeated testing of both patients and controls is less than 5 per cent (98).

Studies on Different Components of the Immune Response in Patients with Renal Carcinoma

1. Human renal carcinoma contain antigens not present in normal adult renal tissue (88–97).
2. The antigens are immunogenic in the autologous host leading to TCMH which can be detected *in vitro* (89, 96).
3. Renal carcinomas of the same histological type contain common antigens, which means that allogeneic renal carcinoma tissue extracts can be used for preoperative evaluation of TCMH (89–92, 96).
4. Allogeneic hypernephroma tissue extracts induce a dose-related leucocyte migration inhibition in patients with non-metastatic renal carcinoma (90, 91).
5. TCMH against allogeneic hypernephroma tissue extracts is present significantly more often in renal carcinoma patients without distant metastases than in patients with metastases (89, 93, 96).
6. The antigens in renal carcinomas may be of foetal origin or contain foetal components, since it can be shown that patients with renal carcinoma are sensitized towards antigens in extracts of foetal kidney tissue (89, 96).
7. Sensitization towards foetal kidney tissue extracts is present significantly more often in renal carcinoma patients without distant metastases than in patients with metastases (89, 96).
8. TCMH against allogeneic hypernephroma tissue extracts is present significantly more often in renal carcinoma patients without distant metastases than in patients with, because:
 - the antigens in metastatic renal carcinoma may be blocked by antigen-antibody complexes or tumour-spe-

cific antibodies as it can be shown that the capacity of renal carcinoma tissue extracts to induce migration inhibition is inversely correlated to the clinical stage of the tumour donor (93),

- extensive leucocyte washings *in vitro* can remove a factor (circulating tumour antigen?) from the cells which inhibits their reactivity *in vitro* (95),
 - serum from patients with renal carcinoma is capable of inhibiting the reactivity of leucocytes from patients with renal carcinoma against allogeneic hypernephroma tissue extracts *in vitro*, indicating the presence of tumour antigen in serum (88),
 - some patients with renal carcinoma have a defective GIC as judged by their lymphocyte ability to react to common antigens and mitogens *in vitro* (97).
9. In patients with renal carcinoma evidence of
- localized tumour,
 - TCMH against allogeneic hypernephroma tissue extracts,
 - sensitization against foetal kidney tissue extracts,
 - low degree of blocking or inhibiting activity,
- are good prognostic signs at the time of initial treatment. Moreover, it is possible to isolate a high-risk group among renal carcinoma patients without clinically detectable distant metastases, but without evidence of TCMH (96).
10. This seems to indicate that the antigens detected *in vitro* by means of LMCT are target in the immunological elimination of tumour cells *in vivo* (96).

Critical Factors in the Study of TCMH by Means of the LMCT

Several factors seem to be critical for the outcome of the interaction between leucocytes and tissue extracts using the LMCT in cancer patients:

I. *Tissue extracts.*

- 1) the protein concentrations.
- 2) the antigenicity of the extracts.

II. *Patients.*

- 1) the clinical stage at the time of testing.
- 2) the sensitivity of the cell donor.

III. *The methods used for determining significant leucocyte migration inhibition.*

Most leucocyte migration studies in cancer patients have neglected several of these factors (Table 3).

I. *Tissue Extracts*

The dose-related effect of tumour extracts on the *in vitro* migration of leucocytes as described by Kjaer (90, 91) makes it of utmost importance to perform extensive extract titration experiments in both patients and controls. Apart from the studies of Kjaer few investigations have included extensive titrations (54, 109, 111, 112). Most workers have used one or two protein concentrations, and some do not comment on the protein concentration used at all (Table 3). Other studies have used enormous protein concentrations (123, 158–160) without reporting on the reactivity of control patients. Thus, it is not possible to judge whether the protein concentrations used were optimal. Moreover, since the range of concentrations revealing a specifically altered reactivity in patients as compared with controls may be extremely narrow (90), it is not possible to evaluate whether such results are specific at all. The problem of leucocyte migration stimulation likewise is apparent in several studies without being commented on at all. As earlier discussed in detail by Kjaer (90) this may be indicative of weak sensitization, but may also be caused by the use of too low protein concentrations. Therefore clarification of this point is important.

Regarding tumour antigenicity, i.e. the effect of individual extracts to induce leucocyte migration inhibition in allogeneic combinations (93), some communications have noted differ-

ences in the efficiency of different preparations (111, 112), but apart from the studies of Black et al. (19–21) and Kjaer et al. (93) no approaches have been made to evaluate systematically this most important aspect. In some studies extracts have been used from metastatic lesions. In view of the differences in antigenicity of disseminated and non-disseminated renal carcinoma (93) no effect of such extracts could be expected.

II. *Patients*

Several papers report on stage-related decline in migration inhibition with advancing tumour burden (Table 3). A number of studies, however, have been unable to find such a relationship. If one wants to register the occurrence of TCMH by means of the LCMT it is of importance to examine patient materials as homogeneous as possible with regard to clinical stage and the use of well-defined timing of tests in relation to treatment (i.e. surgery), for instance, series of patients with minimal tumour burden in the preoperative stage. Patient materials comprising various tumour types (Table 3) have limited value, since great differences between tumour types may be expected as shown in malignant melanoma and breast carcinoma by Cochran et al. (37). Stage-relation of TCMH was found in both types, but involvement of regional lymph nodes was far more important for the outcome of the test among patients with melanoma than among patients with breast carcinoma. Patients undergoing other modalities of treatment than conventional (i.e. cytostatics, immunotherapy) should not be included as such therapy modify the responses.

Except for the studies of Kjaer (93, 95–97) no investigations have aimed at quantitating the degree of TCMH in each individual patient. This grading is of importance, because patients with reactivity defined as significant migration inhibition (89, 90, 96) vary widely in their responses even if the other factors, 1) type of tissue extract, 2) protein concentration, 3) antigenicity of the extracts, and 4) clinical stage of

the patients are constant. This makes it possible to isolate patients with high or low degree of hypersensitivity, which may have significant implications as regards prognosis and response to therapy (96).

III. *The Methods used for Evaluating the Results of Leucocyte Migration Inhibition Tests*

Significant leucocyte migration inhibition may be calculated in two different ways:

- 1) Non-parametrically, comparing groups of results (94, 95).
- 2) By evaluation of individual migration indices in relation to normal ranges.

Almost all leucocyte migration studies of TCMH indicate the proportion of patients with a significant response (Table 3). Most studies, however, use arbitrary cut-off limits of significant migration inhibition, most often migration indices of less than 0.80.

The basis for using normal ranges at all is a determination of the dose-related effect of tissue extracts on leucocytes from patients as well as controls (90-93, 96). The normal range used should be calculated on the basis of the mean value ± 2 s.d. of the migration indices in the control groups using the maximal non-toxic concentrations of tissue extract (89, 92, 96) as it can be shown that this normal range varies with the protein concentration of the extracts, the type of extracts used, and the antigenicity of the tumour preparations (90, 91, 93, 98).

Most leucocyte migration inhibition studies performed in cancer patients have used insufficient evaluation of the parameters of importance for the outcome of the results (Table 3).

A Hypothesis Concerning the Relationship between TCMH and the Clinical Course in Patients with Renal Carcinoma

Human renal carcinomas express antigens not present in the corresponding normal renal tissue. The antigens are im-

munogenic, leading to a TCMH detectable *in vitro*. The antigens in different renal carcinomas contain identical components which may be of foetal origin. They seem to be of the rejection type and target in the immunological elimination of tumour cells *in vivo*.

The antigens are most predominantly being expressed in early stages of the disease before systemic dissemination of the tumour has occurred. The antigens are being shed from the tumour cells to the circulation there coating specifically sensitized lymphocytes.

As the tumour progresses TCMH becomes undetectable as a result of a combination of the following factors:

1. The rate of tumour antigen shedding accelerates thereby inhibiting specifically sensitized lymphocytes from eliminating the tumour cells (antigenic inhibition).
2. The antigens in the tumour may be blocked by circulating antigen-antibody complexes or circulating tumour-specific antibodies coating the antigens and thereby preventing the destruction by specifically sensitized lymphocytes (blocking). Moreover significant antigen depletion may take place in the tumour reducing the amount of "targets" being present.
3. As a secondary phenomenon some patients develop a defective general immunocompetence.

The relative role of the different phenomena arbitrating TCMH in the clinical course of individual renal carcinomas is not clear.

Further Investigations

Several important issues still need clarification concerning the role of TCMH and "blocking phenomena" in patients with renal carcinoma:

1. Elucidation of the relative importance of blocking, antigenic inhibition, and antigenic depletion during the clinical course of individual renal carcinomas.

2. Characterization of the "blocking phenomena": Are they results of specific antibodies, immune complexes, or antigenic depletion?
3. Characterization of the antigens present in renal carcinomas using immunochemical techniques.
4. Characterization of the effector cells both in peripheral blood, regional lymph nodes, and within the tumour.
5. Further development of simple direct and indirect migration inhibition techniques making it possible to monitor large numbers of patients employing small volumes of blood.

Studies employing further solubilization of the antigens, development of the indirect two-step-MIF-technique in agarose, and the agarose microdroplet technique are in progress (98).

Most of the investigations performed in human tumour immunology to-day are aimed at two major fields:

1. To find simple and reliable *in vitro* methods by means of which it is possible to monitor different components of the immune response during the clinical course of cancer patients.
2. To find effective methods for stimulating the immune response in cancer patients.

A vast number of investigations concerning different forms of active or passive immunotherapy have been published during the last few years. To perform immunotherapy in cancer patients is based on the assumption that cancer patients are immunodeficient, and that it is possible to correct this immunodeficiency by therapy. However, major points concerning immunological defects in patients with early and advanced cancer need to be clarified. If we do not know in detail the exact sites of immunological defects it is not possible to set up rational schemes for treating the defects. Is the insufficient TCMH in patients with advanced cancer due to antigenic overload, to blocking factors, to decreased tumour antigenicity because of antigenic depletion of the tumour, or to defects in the general immunocompetence of the patients? Studies by Kjaer (88, 93, 95, 96, 97) indicate that all these factors may

play a role in immunological non-reactivity in human renal carcinoma.

The LCMT offers a possibility for a rapid, reliable, reproducible and quantitative assay *in vitro* of TMCH and various functions of the immune system in cancer patients. However, further elucidation of the points raised at the beginning of this section (1-5) is necessary before a rational immunotherapy of cancer patients can be planned, monitored, and thereby critically evaluated.

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Author's address:

Mogens Kjaer
 Medical Department TA 7301
 Rigshospitalet
 18 Tagensvej
 DK-2200 Copenhagen N
 Denmark

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