

## Immunological Studies on the MCA-induced Sarcoma using the MIF Technique\*

Hee-Sup Shin, M.D., Kwang-Ho Rhee, M.D., Ik-Sang Kim, M.D.,  
Woo-Hyun Chang, M.D. and Seung-Hoon Lee, M.D.

*Department of Microbiology, College of Medicine,  
Seoul National University*

### INTRODUCTION

Since George and Vaughan in 1962 observed that peritoneal cells from guinea pigs sensitized to purified protein derivative were inhibited from migration on the glass surface in the presence of the same antigen in comparison with that of cells from control guinea pigs, migration inhibition test (MIF test) has been used frequently as an in vitro correlate of cell mediated immunity (David et al., 1964a; David et al., 1964b; David 1965). In these, sensitized lymphoid cells challenged with antigen in vitro manufacture MIF, and the effect of this lymphokine is measured by its capacity to inhibit migration of monocytes (Feinstone, 1969; Yamamoto, 1970a; Yamamoto, 1970b; Sandok et al., 1971), phagocytic cells from spleen explants (Taubler et al., 1968), mixtures of lymphoid cells from capillary tubes (Friedman et al., 1969; Smith et al., 1972), macrophages from a droplet on the surface of agar gel (Salvin et al., 1973). Cell mediated immunity in human was also assayed in vitro by MIF test with the human blood leukocytes as effector cells (Mogens, 1971; Falk et al., 1971;

Rosenberg et al., 1971).

It has been generally agreed that a tumor has its own specific antigen which is different from that of corresponding normal cells of the host. And the tumor specific antigen has been identified by various methods such as circulating humoral antibody (Wilson et al., 1965; Perlman et al., 1969; MacLennan et al., 1969) and transplantation test between inbred animals (Foley 1953; Prehn et al., 1957; Prehn, 1972) or between hosts and autochthonous tumors (Klein et al., 1960).

Tumor cells and extracts have been shown to inhibit specifically in vitro the migration of suitable cells taken from individuals sensitized to tumor antigens (Kronman et al., 1969; Halliday et al., 1969; Steiner et al., 1969; Halliday, 1971; Halliday, 1972). And it has long been known that sera from animals sensitized to TSA can enhance the growth of syngeneic tumors (Casey, 1941; Kaliss et al., 1952; Kaliss et al., 1956; Voisin, 1971).

To quantify the cell mediated immune response of the host against developing tumors and to assess the effect on the in vitro cell mediated immunity of sera obtained from mice bearing advanced tumors, the authors adopted the MIF methods.

In this article, we report that the MIF method

\*This work was supported by 1976's research grant of Chung-Ang Cancer Institute and CMB research grant No. 73-300-2 (Received for Publication on December 8, 1976)

could be established in our laboratory to such a degree that it could be applied to various systems, and tumor specific antigen of MCA-induced sarcoma in C<sub>3</sub>H/HeN mice could be detected by MIF technique, and also the immune status of mice which received tumor transplants but not yet developed gross tumors was the same as those of mice sensitized with frozen-thawed tumor cells, that is, immunologically reactive to tumor specific antigens.

In addition, we could find that in the presence of enhancing sera the peritoneal cells from sensitized mice were not inhibited from migration as the normal peritoneal cells, while the peritoneal cells from the same sensitized group of mice were inhibited from migration in the presence of antigen *in vitro*.

## MATERIALS AND METHODS

### 1. Preparation of tumor antigens:

The MCA induced tumors which were developed in C<sub>3</sub>H/HeN mice 7-8 weeks after the subcutaneous injection of 1 mg of 20-methylcholanthrene dissolved in 0.05 ml of lard, were removed, minced, trypsinized and washed with phosphate buffered saline (pH 7.4). Cells were counted with hemocytometer and stored in the deep freezer (-50°C).

### 2. Sensitization of mice with antigen:

Age matched 20 mice (C<sub>3</sub>H/HeN, 4 weeks old) were injected with  $5 \times 10^5$  tumor cells prepared by thawing the frozen tumor cells above mentioned, via I.P. 5 times with 1 week intervals. They received some boosters before the test if necessary.

### 3. Media, Solution and Mouse Sera:

HBSS containing 1 unit/ml sodium heparin, 100 units/ml crystal penicillin (C.P.) and 100 mcg/ml streptomycin (S.M.) was used in obtaining the peritoneal cells from mice after

thermoequilibrated in 37°C.

HBSS with the same antibiotics but without heparin was used in washing the cells after being cooled in ice-water chamber. Normal mice sera were obtained by decapitating the C<sub>3</sub>H/HeN mice which received no treatment, sterilized by Millipore filtration and stored at -50°C. Enhancing sera were obtained from mice carrying large tumors (more than 1.5 cm in diameter) through the ophthalmic venous plexuses at the time of experiment. M-199 supplemented with 10% normal or enhancing sera and 100 units/ml C.P. and 100 mcg/ml S.M. was used as the culture media in the MIF chamber and as the washing fluid of the cells in the third washing. Serum was inactivated at 56°C for 30 minutes.

### 4. Peritoneal cells:

Non-stimulated peritoneal cells were obtained by the technique of Barski et al. (Barski et al., 1969; Le Francois et al., 1971; Youn et al., 1973). It can be summarized as follow:

7 ml of HBSS containing antibiotics and heparin was injected into the peritoneal cavity of a mouse through a multiperforated #18-needle attached to a 10 ml syringe. The abdomen was gently massaged and the fluid was aspirated and reinjected three times without removal of the needle. The aspirated fluid was transferred into a graduated conical centrifuge tube which had been plugged with cotton plug and spun down at 900 rpm for 5 minutes.

### 5. Counting the cells:

The cells from the same experimental group were pooled and washed twice with ice-cold HBSS at 900 rpm for 5 minutes. And the turbidity of the cell suspension was determined from spectro-photometric reading at 660nm on side portion of the suspension diluted 1:5 in 3% acetic acid solutions. The final suspensions (3 ml) were swirled just before reading so that the

cells were in motion. A standard curve was used as a reference, prepared from hemocytometer-counted cells(Chase, 1971).

### 6. Migration chamber and coverslips:

Circular plain ground glassrings(ext. diameter 18mm, int. diameter 16mm, height 2.3mm) were mounted upon slide glasses and fixed with silicone grease.

Cover slips(Matsugami glass 22mm×22mm) were used as the cover of the chamber.

### 7. Migration study:

Killed tumor cells were prepared from the frozen stock by thawing and mixed with the peritoneal cells in the ratio of 1:1 or 1:10 after the peritoneal cells being washed with M-199 containing antibiotics and sera. The mixture was incubated at 37°C for 10 minutes (Kronman, 1969).

After then, the mixed cell suspensions were sucked up into capillary tubes(nonheparinized, internal diameter 1.2mm; external diameter 1.4 mm, 75mm in length), and the one end of the capillary tube was sealed with paraffine wax.

The capillary tubes were spun down in sterile screw-capped tubes at 800 rpm for 5 min. The fluid-cell interface was cut down with a diamond pencil and the cell containing portion was placed on the bottom of a chamber and held in place by means of a small spot of silicone grease.

Culture media was slowly infused into the

chamber at the constant flow through a 26-gauge needle and the chamber was covered with a sterile cover-slip caring not to leave any air bubbles in the chamber.

Then the chambers were incubated in a humidified incubator at 37°C, under 5% CO<sub>2</sub> in air for 48 hours (David et al., 1964a; David et al., 1964b; David, 1965).

### 8. Measurement and Calculations:

After 48 hours of incubation, the chambers were projected on the viewing screen attached to the microscope (AO spencer, Microstar) at the magnification of ×220. The area of migration was drawn on the tracing paper and measured by counting the squares included in the area. The 220 fold magnified area is expressed in cm<sup>2</sup>.

Percent inhibition was calculated by the following formula:

$$\frac{A-B}{A} \times 100 = \% \text{ inhibition}$$

A : Area of migration of control peritoneal cell

B : Area of migration of test peritoneal cell

All data were treated by Student-t test.

## RESULTS

1. Non-specific(non-immunological) blocking effect of tumor cells on the migration of peritoneal cells.

When the sensitized cells were mixed with

**Table 1.** Migration inhibition of sensitized peritoneal cells in the presence of tumor cells in the capillary tube

P.C. donor*	T.C.**	Mean area of migration ±SD	% inhibition	Size of Sample
Sensitized mice***	+	8.5±2.60	90%	3
Sensitized mice	-	83.0±7.40	0	3

\* P.C. : Peritoneal cell

\*\* T.C. : Killed tumor cells prepared by thawing the frozen tumor cell

\*\*\* Sensitization schedule; beginning at age of 4 weeks old, 5 times with 1 week intervals, 5×10<sup>5</sup> tumor cells, I.P., boosted at 2 weeks and 6 weeks after the last sensitization. MIF test 4 days after the last booster. p<0.05

**Table 2.** Nonspecific blocking effect of MCA tumor cells on the migration of normal peritoneal cells

T.C./P.C.*	Mean area of migration $\pm$ S.D.	% inhibition	Size of Sample
1/1	69.8 $\pm$ 2.48	32%**	2
1/10	103 $\pm$ 3.61	0	2
0/1	118.5		1

\* P.C. from normal mice

\*\* Because of the smallness of the sample size of the 0/1 mixture the comparison was done between 1/1 and 1/10 mixtures. ( $p < 0.05$ )

tumor antigen, in the ratio of 1:1 they were inhibited from migration on the glass surface 90% in comparison with the sensitized cells without antigen ( $p < 0.05$ ) (Table 1).

To examine the presence of the non-specific (non-immunological) blocking effect of tumor cells on the migration of peritoneal cells, normal peritoneal cells were mixed with tumor cells in varying ratio and migrated. Because of the smallness of the sample size, it would be difficult to say how much inhibited in comparison with peritoneal cells without antigens, but it could be suggested that when mixed with antigen in the ratio of 10:1 the peritoneal cells were not significantly inhibited by the non-specific blocking effect, but when mixed in the ratio of 1:1 they were 32% inhibited, even when compared with the 10:1 mixtures ( $p < 0.05$ ) (Table 2).

2. To see if any difference in results could be observed between the methods of antigenic presentation, tumor antigens were added in the chamber media instead of mixing directly with

the peritoneal cells in capillary tubes. The area of migration of the sensitized peritoneal cells were 8.05 with tumor antigen and 21 without tumor antigen. That is, the sensitized peritoneal cells in capillary tubes were 74% inhibited from migration when the tumor cell density was more than  $4 \times 10^5$ /chamber in comparison with the sensitized cells without tumor cell ( $p < 0.05$ ).

When the tumor cell density was more diluted as to be  $4 \times 10^4$  or  $4 \times 10^3$ /chamber they were not inhibited (Table 3).

3. To examine the host's cell mediated immune response to the frozen-thawed tumor cells, peritoneal cells from sensitized mice and those from normal mice were mixed with tumor cells respectively in the ratio of 10:1, and filled into capillary tubes and incubated. The area of migration of sensitized peritoneal cells with tumor cells was 19.6 and that of normal peritoneal cells with tumor cells was 59.7. So the sensitized peritoneal cells were 67% inhibited from migration by the tumor cell antigen in comparison with the normal peritoneal cells

**Table 3.** Migration inhibition of sensitized peritoneal cells in the presence of tumor cells in the chamber

P.C. donor	T.C. density* per plate	Mean area of Migration $\pm$ SD	% inhibition	Size of Sample
Sensitized mice	$4 \times 10^5$ /plate	8.05 $\pm$ 1.41	74%**	3
Sensitized mice	0	21 $\pm$ 12.03	0	2

\* When the density of killed tumor cell was more diluted (eg,  $4 \times 10^4$  or  $4 \times 10^3$ /chamber) there was no inhibition. Tumor cells were added into the chamber media directly instead of mixing with the peritoneal cells in capillary tubes.

\*\*  $p < 0.05$

**Table 4.** Sensitized Cells vs. Normal Cells

P.C. donor	T.C./P.C.*	Mean area of migration $\pm$ S.D	% inhibition	Size of Sample
Sensitized mice	1/10	19.6 $\pm$ 12.26	67**	4
Normal mice	1/10	59.7 $\pm$ 16.25	0	3

\* Tumor cells were mixed with peritoneal cells in the ratio of 1:10 and filled into capillary tubes.

\*\* p<0.05

(p<0.05)(Table 4).

4. Cell mediated immune response of a host against transplanted tumor cells before a gross tumor develops.

Peritoneal cells from a group of mice which were injected  $5 \times 10^6$  viable tumor cells via S.C. but not yet developed grossly visible or palpable tumors, were obtained and compared with peritoneal cells from sensitized mice with frozen-thawed tumor cells or peritoneal cells from normal mice.

In this experiment the area of migration of the peritoneal cells from mice which had received tumor transplants was 27.7, that of the peritoneal cells from sensitized mice was 23.8 and that of the normal peritoneal cells were 45.0. So the peritoneal cells from mice transplanted with tumor cells were 38.7% inhibited in comparison with the normal peritoneal cells and the sensitized peritoneal cells were 47.1% inhibited(Table 5).

**Table 5.** MIF response of host's peritoneal cells to killed tumor cells before a grossly visible or palpable tumor develops after tumor cell transplantation

P.C. donor	T.C./P.C	Mean area of migration $\pm$ SD	% inhibition	Size of Sample
Sensitized mice	1/1	23.8 $\pm$ 6.64	47.1**	4
Tumor cell transplanted mice	1/1	27.9 $\pm$ 9.14	38.7*	7
Normal mice	1/1	45.0 $\pm$ 8.2	0	4

\* Received  $5 \times 10^6$  viable tumor cells subcutaneously on the dorsum 13 days before the experiment. No visible or palpable tumor developed until this experiment. p<0.05

\*\* p<0.05

5. Effect of enhancing sera on the migration of peritoneal cells from immunized mice. To

examine the blocking effect of sera obtained from mice bearing large tumors (more than 1.5cm

**Table 6.** Effect of enhancing sera on the migration of sensitized peritoneal cells

P.C. donor	T.C./P.C.	M-199 supplemented with	Mean area of migration $\pm$ SD	% inhibition	Size of Sample
Sensitized mice	1/1	10% Normal sera	12.8 $\pm$ 5.83	51.5%***	6
Sensitized mice	1/1	10% Enhancing sera*	22.6 $\pm$ 10.21	14 %**	8
Normal mice	1/1	10% Normal sera	26.4 $\pm$ 10.48	0	4

\* M-199 supplemented with 10% enhancing sera was used in the last washing of the pooled peritoneal cells and as the chamber media.

\*\* Inhibition of migration of the sensitized peritoneal cells was totally abrogated by substitution of the normal mice sera with enhancing sera. Non-significant, statistically.p>0.05

\*\*\* p<0.05

in diameter), sensitized peritoneal cells were divided into two, and the one portion was tested with M-199 supplemented with 10% normal sera as in the above experiments and the other portion was tested with M-199 supplemented with 10% enhancing sera in the procedures after the second washing with HBSS. And they were mixed with tumor cells separately. The migration area of the former was 12.8 and that of the latter was 22.6 while that of normal peritoneal cells was 26.4.

Sensitized peritoneal cells were inhibited significantly (51.5%, at  $p < 0.05$ ) when tested with M-199 supplemented with normal sera but were nonsignificantly inhibited when tested with M-199 supplemented with enhancing sera (14%, at  $p > 0.05$ ).

## DISCUSSION

In the process of detecting the antigenicity of MCA-induced tumor in  $C_3H/HeN$  mice, MIF technique with mammalian cellular antigen has been established.

Kronman et al.(1969) reported that the sensitized peritoneal cells were inhibited from migration only when the peritoneal cells were mixed with tumor cells at the ratio of 1:1 in capillary tubes. But in this experimental system, inhibition could be obtained also when the peritoneal cells to the tumor cells was 10:1.

Furthermore, the MIF test could be effectively performed by adding the tumor cells directly into the chamber media rather than mixing the peritoneal cells in the capillary tubes.

These two modifications would make contributions to lessen the non-specific blocking effect on migration exerted by the tumor cells in the capillary tube and to make it possible to save the laborious work of counting the peritoneal cells.

Because there was general variability in

the migration between the experiments performed at different time, control group must be included in every experiment. As the cause of the variability, the delay of time in vitro, some differences such as in pH between the batches of solution and media, cleanness of the glassware, contact with heat during the various aseptic procedures and undetected biological differences of the peritoneal cell donors could be supposed.

Anyway, basic experimental steps to establish the MIF technique is completed and we suppose that this technique could be applicable to clinical problems such as the followings; diagnosis of organ specific autoimmune diseases by detecting the CMI response to autologous tissue, host's CMI response to transplanted tissues which may be most important in graft rejection and detection of recurrences of surgically removed tumors and the evaluation of the process of restoration of the CMI function in patient with tumor or patient who had received operation of the tumor resection.

The new antigen produced by the tumor cells may be recognized and eliminated by the host's immune surveillance mechanism. But when the tumor manages to escape the surveillance mechanisms it grows to a detectable size(Mitchison, 1975).

When the host immune surveillance mechanism is normally operating, the host may perform normal immune response (Burnet, 1970; De-Cosse, 1968), and in our experiment we can show that peritoneal cells from mice in these state are inhibited from migration as effectively as the cells from the sensitized mice. This may be explained as that the immune system of the host is actively reacting to the growing tumor before the tumor grows large enough to be palpable or visible.

Since mice have circulating anti-tumor antibodies(MacLennan, 1969; Wilson, 1965; Perlman

et al., 1969; MacLennan, 1970a & 1970b), and possibly other factors both during tumor progression and loss of tumors (Halliday, 1972; Bernstein et al., 1972; Baldwin, 1972), it is interesting to examine the effect of the serum on the MIF test. In the experiment using the so-called enhancing sera, we obtained results which show that the migration inhibition of the peritoneal cells from the sensitized mice is totally abrogated by enhancing sera which is added to the medium and washing fluid for the sensitized peritoneal cells before contact with the tumor cells. This is in accordance with the experiments of others that sera from mice which are bearing large tumors could enhance the tumor graft when injected with the tumor cells (Casey, 1941; Kaliss et al., 1952; Kaliss et al., 1956; Voisin, 1971).

### SUMMARY

It has been generally agreed that in a chemically induced, tumor the tumor specific antigen could be identified by circulating humoral antibody and transplantation test among inbred animals.

To quantify the cellular immunity to the MCA-induced tumor and to assess the in vitro effect of serum of mice which were bearing advanced tumors, the authors adopted the MIF test.

And the results obtained are summarized as follows:

1. MIF technique has been established using the mammalian tumor cells as antigen with the ratio of peritoneal cells to tumor cells as 10:1 or 1:1, and the test could be performed with good results by adding the tumor cells directly into the media of the chamber instead of mixing with the peritoneal cells in the capillary tubes.
2. The antigenicity of the MCA-induced sarco-

ma in C<sub>3</sub>H/HeN mice was detected by MIF technique. When peritoneal cells obtained from mice sensitized with frozen-thawed tumor cells were challenged with the same tumor cells in vitro, they were inhibited significantly from migration on the glass surface as compared with those from normal mice.

3. In the host which had received viable tumor cells but not yet developed a grossly visible or palpable tumor, in vitro cell mediated immune response to the tumor cells (detected by MIF test) was the same as that of actively sensitized host.
4. The blocking effect on the cell mediated immunity in vitro of the sera obtained from mice which were bearing advanced tumors was detected by using the sera as substitute for normal sera in the media of MIF chambers and in the procedure of washing the peritoneal cells before contact with the tumor cells.

### ≫국문초록≪

#### 실험적으로 유발한 동물종양에 있어서 거식세포유주저지법을 이용한 면역학적 연구

서울대학교 의과대학 미생물학교실

신희섭 · 이광호 · 김익상 · 장우현 · 이승훈

화학적으로 유발된 동물종양에 있어서 순계동물간의 종양이식시험이나 혈액중의 항체에 의하여 증명되는 종양 특이 항원이 존재함은 일반적으로 인정되고 있다. MCA유발육종에 대한 숙주의 세포성 면역반응을 측정하고 점진적으로 성장하는 종양을 가진 마우스혈청의 세포성면역 반응에 대한 효과를 생체외에서 검증하기 위하여 거식세포유주저지법을 도입하여 다음과 같은 결과를 얻었다.

1. 종양과 숙주의 관계에서 종양세포에 복강세포의 비율 1:10 또는 1:1로 사용하여 거식세포유주저지법을 정립할 수 있었으며 또한 종양세포를 복강세포와 섞어서 모세유리관에 넣어서 유주시키는 대신에 배지에 직접 넣고서도 본 실험을 효과적으로 수행할 수 있었다
2. 살아있는 종양세포를 이식하여 큰 종양이 발생되

기전의 마우스의 그 종양항원에 대한 세포성면역 반응은 죽은 종양세포로 면역한 마우스의 세포성 면역반응과 같았다.

3. 직경 1.5cm이상의 종양을 가진 마우스의 혈청을 면역된 마우스의 복강세포가 종양세포를 만나기 전에 복강세포에 처리하고 배지에도 첨가했을 때에 면역된 마우스의 복강세포의 유주저지 현상이 무효화되었다.

## REFERENCES

- Baldwin, R.W., Price and R.A. Robins: *Blocking of lymphocyte mediated cytotoxicity for rat hepatoma cells by tumor specific antigen-antibody complex. Nature New Biology*, 238: 185, 1972.
- Barski, C. and J.K. Youn: *Evolution of cell-mediated immunity in mice bearing an antigenic tumor. Influence of tumor growth and surgical removal. J. Natl. Cancer, Inst.*, 43: 111, 1969.
- Bernstein, I.D., D.E. Thor, B. Zbar and H.J. Rapp: *Tumor immunity: Tumor suppression in vivo initiated by soluble products of specifically stimulated lymphocytes. Science*, 172: 729, 1972.
- Burnet, M: *Immunological surveillance. Pergamon Press, Oxford. p.161, 1970.*
- Casey, A.E.: *Experiments with a material from the Brown-Pearce tumor. Cancer Res.*, 1: 134, 1941.
- David, J.R.: *Suppression of delayed hypersensitivity in vitro by inhibition of protein synthesis. J. exp. Med.*, 122: 1125, 1965.
- David, J.R., H.S. Lawrence and L. Thomas: *Delayed hypersensitivity in-vitro. II. Effect of sensitive cells on normal cells in the presence of antigen. J. Immunol.*, 93: 274, 1964.
- David, J.R., S. Al-Askari, H.S. Lawrence and L. Thomas: *Delayed type hypersensitivity in-vitro, I. The specificity of inhibition of cell migration by antigens. J. Immunol.*, 93: 264, 1964.
- De-Cosse, J.J. and S. Gelfant: *Noncycling tumor cells: Mitogenic response to antilymphocytic serum. Science*, 162: 998, 1968.
- Falk, R.E. and J.B. Zabriskie: *The capillary technique for measurement of inhibition of leukocyte migration. An assessment of cell-mediated immunity. In In-vitro methods in cell-mediated immunity, ed. by Barry R. Bloom and Philip R. Glade, Academic Press, New York and London. p.301, 1971.*
- Feinstone, S.M., E.H. Beachey and M.W. Rytel: *Induction of delayed hypersensitivity to influenza and mumps viruses in mice. J. Immunol.*, 103: 844, 1969.
- Foley, E.J.: *Antigenic properties of methylcholanthrene induced tumors in mice of the strain of origin. Cancer Res.*, 13: 835, 1953.
- Friedman, H., M. Sanz, C. Combe, L. Mills and Y. Lee: *Migration of mouse lymphocytes in vitro from capillary tube cultures. Proc. Soc. exp. Biol. Med.*, 132: 849, 1969.
- George, M. and J.H. Vaughan: *In-vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. exp. Biol. Med.*, 111: 514, 1962.
- Halliday, W.J.: *Macrophage migration inhibition with mouse tumor antigens: Properties of serum and peritoneal cells during tumor growth and after tumor loss. Cell. Immunol.*, 3: 113, 1972.
- Kaliss, N. and A.A. Kandutsch: *Acceptance of tumor homografts by mice injected with antiserum. I. Activity of serum fractions. Proc. Soc. exp. Biol. Med.*, 91: 118, 1956.
- Kaliss, N. and N. Molomut: *The effect of prior injections of tissue antiserum on the survival of cancer homografts in mice. Cancer Res.*, 12: 110, 1952.
- Klein, G., H.O. Sjögren, E. Klein, and K.E. Hellström: *Demonstration of resistance against methylcholanthrene induced sarcomas in primary autochthonous host. Cancer Res.*, 20: 1516, 1960.
- Kronman, B.S., H.T. Wepsic, W.H. Churchill, Jr., B. Zbar, T. Borsos, H.J. Rapp: *Tumor-specific antigens detected by inhibition of macrophage migration. Science*, 169: 296, 1969.
- Le Francois, D., Youn, J.K., Belehradek J. Jr. et al.: *Evolution of cell mediated immunity in mice bearing tumors produced by a mammary carcinoma cell line. Influence of tumor growth, surgical removal and treatment with irradiated tumor cells. J. Natl. Cancer Inst.*, 46: 981, 1971.
- MacLennan, I.C.M., G. Loewi and A. Howard: *A human serum immunoglobulin in lymphocyte mediated cell damage in vitro. I. Comparison of the effects of target cell specific antibody and normal serum*



- factors of cellular damage by immune and non-immune lymphocytes. *Immunol.*, 18: 397, 1969.
- MacLennan, I.C.M. et al: *The role of immunoglobulin in lymphocyte mediated cell damage in vitro. II The mechanism of target cell damage by lymphoid cells from immunized rats. Immunol.*, 18: 405, 1970.
- Mogens Söborg: *The leukocyte migration technique for in-vitro detection of cellular hypersensitivity in man. In vitro methods in cell-mediated immunity*, edited by Bloom and Glade Academic Press, New York, p.289, 1971.
- Mitchison, N.A.: *Immunology of cancer in animals. In Clinical Aspect of Immunology*, 3rd ed., edited by Gell, Coombs and Lachmann, Blackwell Scientific Publications, p.601, 1975.
- Perlman, P. and G.Holm: *Cytotoxic effect of lymphoid cells in vitro. Adv. Immunol.*, 11: 117, 1969.
- Prehn, R. T.: *The immune reaction as a stimulator of tumor growth. Science*, 176: 170, 1972.
- Prehn, R.T. and Main, J.M.: *Immunity to methylcholanthrene-induced sarcomas. J. Natl. Cancer Inst.*, 18: 769, 1957.
- Rosenberg, S.A. and J.D. David: *In vitro assay for inhibition of migration of human blood leukocytes. In In-vitro methods in cell-mediated immunity*, edited by Bloom and Glade, Academic Press, New York p.297, 1971.
- Salvin, S.B., Youngner, J.S., and Lederer, W.H.: *Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. Infection and Immunity*, 7: 68, 1973.
- Sandok, P.L., Ronald D. Hinsdill, and R.M. Albrecht: *Migration inhibition of mouse macrophages by Brucella antigens. Infection and Immunity*, 4: 516, 1971.
- Smith, R.A. and Nancy J. Bigley: *Detection of delayed hypersensitivity in mice injected with ribonucleic acid-protein fractions of Salmonella typhimurium. Infection and Immunity*, 6: 384, 1972.
- Taubler, J.H. and Stuart Mudd: *Staphylococcal delayed hypersensitivity in mice, II. In vitro demonstration and specificity of delayed hypersensitivity. The Journal of Immunology*, 101: 550, 1958.
- Voisin, G.A.: *Immunological fascilitation, a broadening of the concept of the enhancement phenomenon. Progress in Allergy*, 15: 328, 1971.
- Wagner, J., M. Feldman, W. Boyle and J.W. Schrader: *Cell mediated immune response in vitro. J. exp. Med.*, 136: 331, 1972.
- Wilson, D.B.: *Quantitative studies on the behavior of sensitized lymphocytes in vitro. J. exp. Med.*, 122: 143, 1965.
- Yamamoto, K. and R.L. Anacker: *Macrophage migration inhibition studies with cells from mice vaccinated with cell walls of Mycobacterium bovis, BCG: Characterization of the experimental system. Infection and Immunity*, 1: 587, 1970.
- Yamamoto, K., R.L. Anacker and E. Ribi: *Macrophage migration inhibition studies with cells from mice vaccinated with cell walls of Mycobacterium bovis BCG: Relationship between inhibitory activity of lung cells and resistance to air borne challenge with Mycobacterium tuberculosis H37Rv. Infection and Immunity*, 1: 595, 1970.
- Youn, J.K., Le Francois, D., Barski, G.: *In vitro studies on mechanism of the "eclipse" of cell-mediated immunity in mice bearing advanced tumors. J. Natl. Cancer Inst.*, 50: 921, 1973.