Yu-hui Chang and Chin-sheng Yao

Department of Immunology, Institute of Cancer Research, Chinese Academy of Medical Sciences, Peking

Investigation of the human macrophage II. The *in vitro* cytotoxicity of macrophages

Macrophage-mediated cytotoxicity *in vitro* was studied by a tritiated thymidine incorporation inhibition assay as well as by microscopic examination and optical transmittance determination. It was found that macrophages from patients with malignant diseases showed cytotoxic effects on two malignant human cell lines. The cytotoxic activity was more marked in patients who were clinically tumor-free. Some patients with benign diseases and normal subjects also exhibited cytotoxic macrophages. Macrophage-mediated cytotoxicity (MMC) was thus nonspecific in nature. Cell-free exudate from cancer patients did not influence MMC appreciably. The experimental methods and the possible significance of MMC are discussed.

1 Introduction

Although many investigations have shown that phagocytosis is the most characteristic feature of the macrophage, its ability to destroy allogeneic cells and abnormal cells actually depends mostly on cytotoxic activity, probably of a nonphagocytic nature. In animal tumor systems, it has been demonstrated that the activated macrophage possesses the ability to suppress and kill tumor cells *in vitro* but does not have this activity against normal cells [1–3]. We have carried out studies of the cytotoxic action of human macrophages against human malignant cells *in vitro*. Preliminary results are presented in this report.

2 Materials and methods

2.1 Harvest and counting of macrophages

The methods employed have been described in the preceding paper [4].

2.2 Target cells

EC-109 cell line of esophageal cancer was established back in 1973 by the Department of Cell Biology of this Institute from a surgical specimen of a patient with squamous cell carcinoma of the esophagus. EC-109 was grown for 4 days in TC 199 medium containing 20% newborn calf serum (NBCS) when the culture medium was changed. After it was grown for three more days, the cell sheath was digested with 0.02% EDTA-0.5% trypsin in a proportion of 3:1 and resuspended in TC 199 with 20% NBCS so that suspensions with 1×10^4 and 5×10^4 single cells per ml were ready for use.

MA cell line of osteogenic sarcoma was established in 1973 by the Department of Virology of this Institute from a surgical specimen of a patient with osteogenic sarcoma. The passage of this cell line and the preparation of cell suspension were similar to those for EC-109.

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Correspondence: Yu-hui Chang, 1, Ya Bao Street, Department of Immunology, Institute of Cancer Research, Chinese Academy of Medical Sciences, Peking, People's Republic of China

Abbreviations: **NBCS:** Newborn calf serum **E/T:** Effector-to-target cell **CI:** Cytotoxic index **CFE:** Cell-free exudate **[^{3}H]dThd:** Tritiated thymidine **MMC:** Macrophage-mediated cytotoxicity **OTM:** Optical transmittance

2.3 *In vitro* cytotoxicity test

2.3.1 Tritiated thymidine ([^{3}H]dThd) incorporation inhibition assay

This was performed according to the method described by Williams et al. [5] with some modification. Macrophages were obtained from skin blister by the method previously described. According to the cell count, an appropriate amount of blister fluid was added to flat-bottomed tubes so that 5×10^4, 10×10^4 and 20×10^4 macrophages were present in the culture tubes, respectively. To each of these tubes was added TC 199 containing 20% NBCS, penicillin (100 units) and streptomycin (100 μg/ml) and the content in each tube adjusted to 1 ml. The cells were incubated at 37 °C (5% CO_2 in air) for 24 h, the supernatant removed and each tube washed with isotonic saline to remove nonadherent cells. One ml of target cell suspension was then added so that the effector-to-target cell (E/T) ratios were 5, 10 and 20, respectively. Each experiment was set up in triplicate. The cells were incubated for 72 h. Sixteen h before termination of culture, [^{3}H]dThd, 1 μCi in 0.1 ml, was added ([Me-^{3}H]dThd, 24 Ci/mmol = 888 GBq/mmol, Institute of Atomic Research, Shanghai). At the end of cultivation, the supernatant fluid was decanted and the cells washed three times with isotonic saline and finally once with absolute ethyl alcohol. Scintillation counting was carried out by the method previously described [4] and the results expressed as cytotoxic index (CI) according to the formula:

$$CI = 1 - \frac{\text{Average amount of [}^3\text{H]dThd incorporated (cpm) into target cells in the presence of macrophages}}{\text{Average amount of [}^3\text{H]dThd incorporated (cpm) into target cells in the absence of macrophages}}$$

In order to study the influence of humoral factors on macrophage-mediated cytotoxicity (MMC), autologous cell-free exudate (CFE) was added to some of the cultures with a final concentration of 10 or 20%. Other conditions remained identical with those in experiments without CFE.

Forty-eight cases of malignant tumors, including 33 cases of esophageal cancer, and 14 cases of benign diseases were examined in this way for their MMC *in vitro*.

2.3.2 Microscopic examination and optical transmittance (OTM) determination

Macrophages were harvested as described and inoculated into the wells of microtest plates (Falcon Plastics, Oxnard, CA, No.

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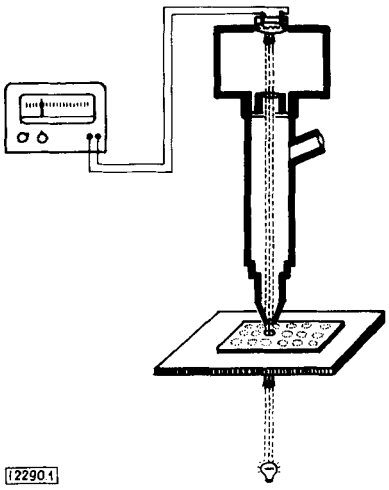

Figure 1. Apparatus for the determination of OTM.

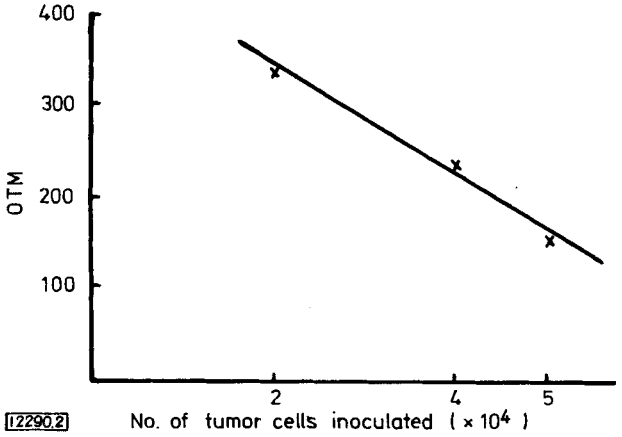

Figure 2. OTM of wells inoculated with different numbers of target cells.

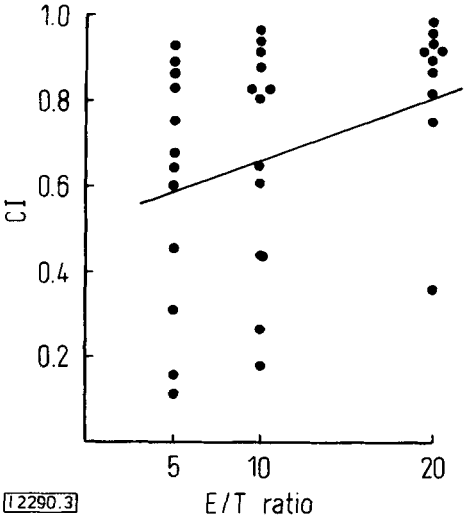

Figure 3. Dose response curve of macrophage-mediated cytotoxicity by [^{3}H]dThd incorporation inhibition assay.

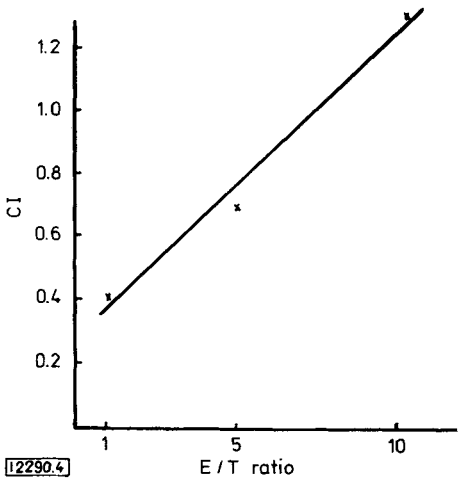

Figure 4. Dose response curve of MMC by OTM determination.

3040), 5×10^4, 25×10^4 and 50×10^4 cells per well. After 24 h incubation in TC 199 with 20% NBCS, the wells were washed with isotonic saline to remove nonadherent cells. To each well were then added 5×10^4 tumor cells (EC-109) so that the E/T ratios were 1, 5 and 10, respectively. Cultivation was continued up to 96 h. After washing, fixation and staining with Giemsa, each well was observed under a microscope and microphotographs taken. The magnitude of cytotoxicity could be judged by the amount of tumor cells left in the well as compared to that of control. To make it more objective, a simple way of scoring cytotoxicity was adopted as shown in Fig. 1. The OTM of each well was determined through a microscope by a photoelectric cell connected to a galvanometer. When the number of cells in the well increased, a proportional decrease of OTM was observed (Fig. 2). CI was calculated from the formula: CI = (A-B)/B where A = average OTM of wells inoculated with tumor cells and macrophages and B = average OTM of wells inoculated with tumor cells only. Using this method, 49 subjects were examined. There were 8 normal subjects and 41 cancer patients (33 cancer of esophagus).

3 Results

3.1 The effect of varying E/T ratios

In our experiments, three different E/T ratios were used. The results shown in Figs. 3 and 4 revealed that, using either method to assay MMC, cytotoxicity increased with the increase in E/T ratios. This indicates that MMC is essentially dose-dependent.

3.2 MMC assayed by [^{3}H]dThd incorporation inhibition

3.2.1 Cytotoxicity against EC-109 cells

Among 33 patients with esophageal cancer surgically treated, 20 were examined before surgery and 13 thereafter. Irrespective of the E/T ratios used, the highest CI was taken as the cytotoxic activity which was considered positive when it exceeded 0.2. As shown in Fig. 5, of esophageal cancer patients 67% gave positive reactions (22/33), among which 13 were examined before and 9 after surgery. Among the negative

reactions, 7 belonged to the group examined before and 4 after surgery. For patients with other types of cancers, including cancer of the gastric cardia, colon, liver, lung, breast etc., the results were practically the same, namely 9 positives in 15 cases (60%). In 14 patients with benign diseases, 6 were positive (42.9%).

3.2.2 Cytotoxicity against MA cells

Five patients with different types of cancers were examined for their MMC against MA cells as well as EC-109 cells. The results (Table 1) revealed that all patients showed cytotoxic reactivity for MA cells, and all but one (ostogenic sarcoma patient) were cytotoxic against EC-109 cells.

3.2.3 Effect of CFE on the cytotoxic activity of macrophages

From the results presented in Table 2 it can be seen that all the 4 patients with esophageal cancer gave positive MMC against EC-109. In the presence of CFE, regardless of its concentrations (10 or 20%) or its origin (from treated or untreated cases), cytotoxicity was practically unchanged, except in one instance where the post-treatment CFE markedly enhanced cytotoxicity. Blocking effect of CFE was never seen.

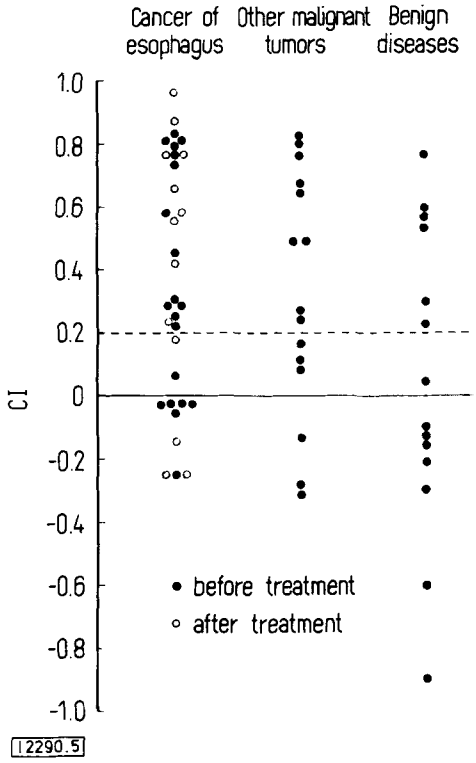

Figure 5. Results of MMC by [^{3}H]dThd incorporation inhibition assay.

Table 1. Cytotoxicity of macrophages against EC-109 and MA cells

Patient	Diagnosis	CI against EC-109	CI against MA
K	Cancer of esophagus	0.31	0.74
W	Osteogenic sarcoma	0.16	0.69
C	Lung cancer	0.82	0.75
L	Cancer of gastric cardia	0.29	0.63
Y	Liver cancer	0.68	0.62

Table 2. Effect of CFE on MMC in patients with esophageal cancer

Patient	Before or after treatment	CFE (%)	E/T ratio	CI
C	Before	20	20	0.95
		–		0.84
F	After	20	20	0.95
		–		0.89
Zh	Before	10	10	0.41
		–		0.42
W	After	10	10	0.79
		–		0.22

Table 3. MMC assayed by OTM determination

Group	No. of positive MMC/No. tested	MMC (mean ± SE)
I Normal subjects	7/8	0.342 ± 0.047
II Cancer patients, before treatment	10/16	0.413 ± 0.116
III Cancer patients, immediately after radiotherapy	8/12	0.484 ± 0.160
IV Cancer patients, clinically tumor-free	11/13	0.636 ± 0.146

3.3 MMC assayed by microscopic examination and OTM determination

An illustrative case is shown in Fig. 6. In the control well (Fig. 6a), multilayers of tumor target cells covered up the whole bottom of the well which resulted in an OTM of 163. When macrophages were added in an E/T ratio of 1 (Fig. 6b) or 5 (Fig. 6c), OTM increased to 230 and 328 and their CI were 0.41 and 0.68, respectively. Fig. 6d is a higher magnification of Fig. 6c, in which can be seen ramnent tumor cells surrounded by many macrophages. The overall results are presented in Table 3. It is evident that macrophages from normal subjects (group I) were mildly cytotoxic. CI of those from cancer patients before treatment (group II) was only slightly higher than that of the normal control ($p > 0.5$). Four patients were examined before and immediately after radiotherapy for esophageal cancer; 3 of them showed much higher cytotoxic activity after therapy. In group III as a whole, however, CI remained unchanged. This seems to indicate that radiotherapy did not influence MMC appreciably. Thirteen patients who were clinically tumor-free for 1 month to 4 years (group IV) gave apparently higher CI values, but the difference between group II and group IV was not significant.

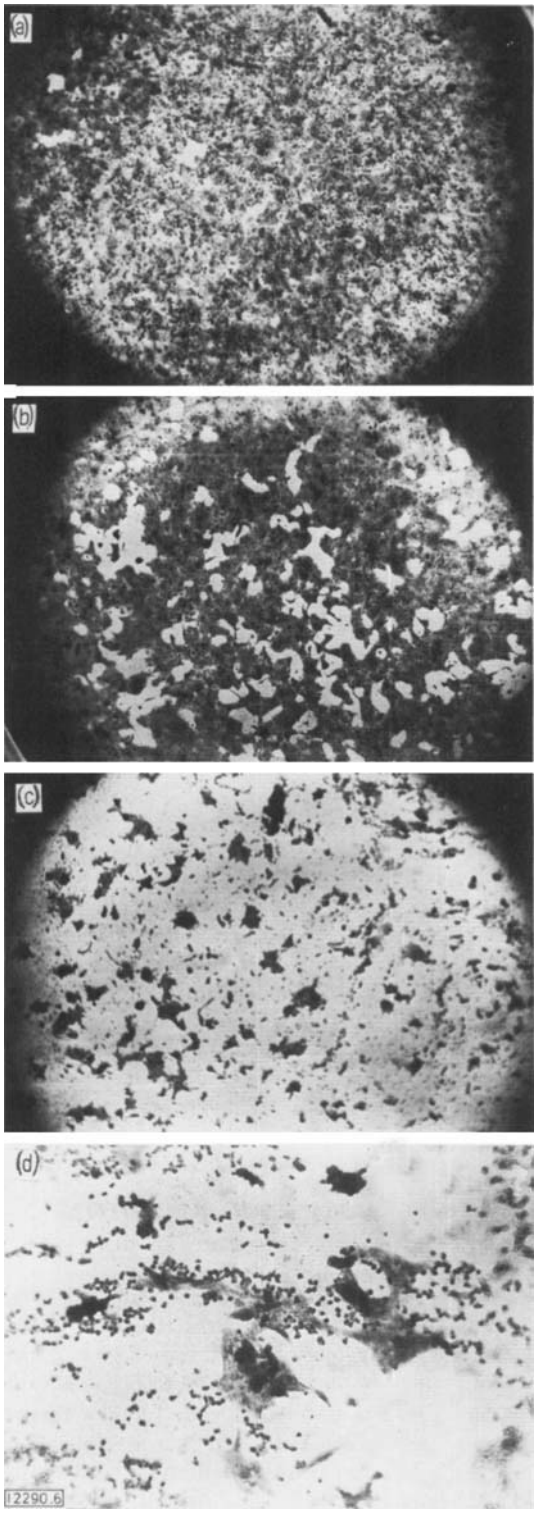

Figure 6. Microphotographs showing MMC *in vitro*. (a) Control well inoculated with tumor cells only; (b) tumor cells cocultured with macrophages, E/T ratio = 1; (c) same as (b), E/T ratio = 5; (d) higher magnification of (c).

4 Discussion

In a [^{3}H]dThd incorporation inhibition assay, we have been able to demonstrate that macrophages of human origin are cytotoxic to human tumor cell lines *in vitro*, *i.e.* inhibition of tumor cell proliferation. We first placed a definite amount of blister fluid into flat-bottomed tubes and then made it up to 1 ml with TC 199. The tubes were incubated for 2 h. Then, the supernatant was poured off, and the nonadherent cells, chiefly lymphocytes, were removed by two successive washings with isotonic saline. Target cell suspensions were then added. The granulocytes, which also adhered to the bottom surface with macrophages, died off on further cultivation, leaving the macrophages to carry out the cytotoxic effect. However, Takasugi and co-workers [6] have reported that granulocytes and their sonic extracts could detach adherent target cells. In our procedure, the culture tube was washed to remove free [^{3}H]dThd. Thus, the question arises whether the decomposed products of granulocytes could bring about detachment of target cells which were washed off by saline. We have noted that the number of granulocytes varies a great deal in different samples of blister fluid and bears no relationship to the magnitude of inhibition of thymidine incorporation (Table 4). Furthermore, according to Takasugi et al., heparin has the ability to inhibit the activity of granulocytes and their sonic extracts on target cell detachment. So we made parallel studies to ascertain whether heparin could exert such an effect, but the results were negative (Table 4). In order to rule out any possible effect due to granulocytes or their products, we have, in subsequent experiments, prolonged the pre-incubation time of the blister fluid to 24 h so that any cytotoxic activity should be attributed to that of the macrophages alone, as all the granulocytes and their products would have been completely removed before the addition of target cells. From studies on animals, it has been reported that macrophages cultured *in vitro* for more than 18 h showed decreased cytotoxic activity [7]. Our results, however, indicated that even when cultured longer than this period, MMC remained unchanged (Table 5). This seems to

Table 4. Influence of heparin on the cytotoxic activity of macrophages

Target cell (1×10^4)	Macrophages (1×10^5)	Granulocytes $\times 10^{-5}$	E/T ratio	Heparin (U)	[^{3}H]dThd (cpm)	CI
EC-109	Patient T	3.8	10	–	1576	−0.12
			10	30	877	0.06
EC-109	Patient Zh	1.6	10	–	1629	−0.16
			10	30	776	0.17
EC-109	Patient S	2.8	10	–	1399	0.01
			10	30	918	0.015
EC-109				–	1401	
				30	928	

Table 5. Effect of duration of cultivation on cytotoxic activity of macrophages

Patient	Diagnosis	CI before culture	CI after culture (days)
H	Intrathoracic goiter	0.24	0.26 (7)[a]
S	Lung cancer with brain metastasis	−0.13	−0.13 (3)
Y	Lung cancer	–	0.44 (3)

a) Cultured in 50% CFE in TC 199.

support our opinion that human macrophages cultured *in vitro* preserve their original physiological activity.

One of the major criticisms of using the [^{3}H]dThd post-label method to assess MMC is that thymidine has been reported [8] to be a normal secretory product of *in vitro* cultured macrophages. Therefore, any decrease in [^{3}H]dThd incorporation into the DNA of co-cultured tumor cells may well be considered to be the result of competitive inhibition by cold thymidine rather than a real cytostatic effect of macrophages. To rule out this possibility, though remote, MMC was performed in an alternative way, and cytotoxicity was quantitated by OTM of wells inoculated with tumor cells with and without macrophages. The results demonstrated that macrophages, in our experimental conditions, did show cytotoxicity against tumor cells *in vitro*. The magnitude of MMC seems to depend on whether patients are clinically tumor-free, and radiotherapy has little, if any, suppressive influence on MMC. According to the results thus obtained, the *in vitro* cytotoxic action of macrophages against cancer cells is nonspecific in nature. In patients with cancers of various histological types, macrophages were cytotoxic to both target cell lines used (Table 1). A number of patients with benign diseases, including benign tumors, had cytotoxic macrophages. It is interesting to see that macrophages from normal individuals were also cytotoxic. However, animal experiments have repeatedly shown that macrophages become cytotoxic only after being activated. The cytotoxic potential of normal macrophages may be the result of (a) activation inside the blister, or (b) activation during pre-incubation, perhaps by some unknown factor(s) released from degraded polymorphonuclear leukocytes. This remains to be investigated.

Using the [^{3}H]dThd incorporation method, a proportion of patients gave negative results. This does not seem to be related to humoral factors, because (a) there was no humoral factor present in our experimental system and (b) there was no blocking effect observed when CFE was added to the system (Table 2). In some of the negative cases, CI gave negative values which might mean that macrophages not only failed to inhibit but actually promoted tumor cell growth. Its cause and mechanism of action must be further investigated.

Although an *in vitro* cytotoxicity test can reflect, to a certain extent, the function of the effector cells involved, it must be recognized that the *in vitro* conditions are, after all, quite different from those *in vivo*. The lack of correlation between *in vitro* and *in vivo* findings has not infrequently been reported in the literature. Therefore, we have been observing MMC *in vivo*. Two patients with squamous cell carcinoma of the tongue diagnosed by biopsy were treated by weekly injection of autologous blister fluid into the tumor and its vicinity. After several weekly treatments, the tumor was surgically excised, and the histological sections of the specimens before and after local injection treatments were compared. In one case, the post-treatment section showed cancer cell nests surrounded by large numbers of multinucleated giant cells. In the interstitial spaces, there was marked infiltration of lymphocytes and plasma cells. Some of the cancer cell nests were invaded by the giant cells and became disorganized. Tumor cell degeneration of varying degrees was seen. In the other case, although tumor cells could be found on the biopsy specimen for the frozen section at the beginning of surgery, the pathological section from the surgically excised specimen did not show tumor cells at all. Of course, these observations are preliminary, but they might still suggest that macrophages do exert some cytotoxic effect on tumor cells *in vivo*. These experiments are being continued.

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