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SENSITIZATION OF T LYMPHOCYTES IN VITRO BY SYNGENEIC MACROPHAGES FED WITH TUMOR ANTIGENS[1]

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We investigated the interaction between T lymphocytes and macrophages in the in vitro sensitization of lymphocytes against tumor cells. Spleen cells were sensitized in vitro by syngeneic peritoneal macrophages that had been fed with cell-free antigen preparation of syngeneic tumor cells. The sensitized T lymphocytes acquired specific cytotoxic activity in vitro and the capacity to inhibit tumor development in vivo. Allogeneic macrophages, syngeneic fibroblasts, or the antigen preparation by itself were not able to sensitize the lymphocytes against the tumor.

One of the approaches to the study of cell-mediated immunity is the primary induction of sensitization *in vitro* (1). T lymphocytes, which were sensitized *in vitro* against fibroblasts (2), lymphocytes (3), or tumor cells (4–6), acquired specific cytotoxic activity against target cells *in vitro*. The adoptive transfer of lymphocytes that had been sensitized *in vitro* against tumor cells was found to be a useful tool for directing *in vivo* an immune response against the tumor (7).

The role of macrophages in the afferent arm of cell-mediated immune response has been investigated in relatively few systems (8). It was found that macrophages were required for the sensitization of lymphocytes *in vitro* against xenogeneic fibroblasts (9) and for allogeneic mixed leukocyte interactions (10, 11). Macrophages from unprimed animals were found to produce lymphocyte-activating factors (12). Macrophages from primed animals were found to participate in the proliferative response of lymphocytes to BCG (13).

Macrophages were found also to mediate the effector arm of cell-mediated immunity by being cytotoxic to different kinds of target cells (14–16).

The interaction of macrophages with tumor tissue *in vivo* was investigated and an inverse correlation between tumor malignancy and the content of macrophages in the tumor was found (17).

In addition to the role of macrophages in cell-mediated immunity, previous work has shown their importance in humoral immunity (8). It was found that injection of sublethally irradiated mice with macrophages fed with bacterial antigens was much more immunogenic than injection of antigen alone (18).

In the present study we investigated the role of macrophages in the primary induction of lymphocyte sensitization against a syngeneic tumor *in vitro*.

We found that peritoneal macrophages, which had been fed with cell-free preparations of syngeneic tumor cells, were able to sensitize syngeneic lymphocytes against the tumor. The sensitized T lymphocytes manifested specific cytotoxic activity *in vitro* and inhibited tumor growth *in vivo*.

MATERIALS AND METHODS

Animals. Inbred 6- to 10-week-old male or female C57BL/6 and C3H/eb mice were supplied by the Animal Breeding Center, The Weizmann Institute of Science.

Tumors. A) The Lewis lung carcinoma (3LL);[2], a malignant metastasizing tumor which originated spontaneously in a C57BL/6 mouse (19) was maintained by subcutaneous transfer of tumor cells to syngeneic male mice.

B) The KWT, a malignant metastasizing sarcoma which developed spontaneously in C3H/Km mouse (20) was maintained as above in C3H/eb female mice.

C) The L-tumor, a fibrosarcoma induced by 3-methylcholanthrene in a C57BL/6 mouse, was maintained by intramuscular transfer of tumor cells to syngeneic male mice.

Suspensions of tumor cells were prepared by treatment of minced tumor tissue for 30 min with a solution of 0.3% trypsin (hog pancreas, Nutritional Biochemicals Corp., Cleveland, Ohio). The cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, and suspended in Dulbecco's modification of Eagle's medium (EM) (Grand Island Biological Co., Grand Island, N. Y.) for preparation of antigen and for inoculation *in vivo*, or in Waymouth's medium supplemented with 5% calf serum (Grand Island Biological Co.,) (Way) for culturing *in vitro* (target cells or sensitizing monolayers).

Preparation of antigen from tumor cells. Fifty to 100×10^6 3LL tumor cells were suspended in 1 ml of EM. The suspension was frozen in liquid air and thawed in 40°C water eight times. The cell-free preparation obtained was centrifuged in $800 \times G$ for 5 min and the supernatants were used as antigen. Occasionally the antigen was stored in −20°C and thawed before use. Similar preparations obtained from mouse cell lines were found to contain crude membrane fractions and ribosomes (21).

Fibroblasts. Primary monolayer cultures of embryonic fibroblasts were obtained from 11- to 14-day mouse embryos and maintained as described (22). Suspensions of fibroblasts were prepared by treatment of primary cultures with a solution of 0.3% trypsin for 10 min at 37°C. The cells were washed with PBS and suspended in Way for preparation of target cells or sensitizing monolayers.

Sensitization of spleen cells (SC) in vitro

A) Sensitization by antigen-fed macrophages. Macrophages were collected from the peritoneal cavity of mice that had received 5 days earlier 3 ml of thioglycollate solution (Fluid

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[2]Abbreviations used in this paper: 3LL, Lewis lung carcinoma; PBS, phosphate-buffered saline; EM, Dulbecco's modification of Eagle's medium; Way, Waymouth's medium supplemented with 5% calf serum; SC, spleen cells; FCS, fetal calf serum.

Thioglycollate Medium, DIFCO Laboratories, Detroit, Mich.) i.p. The macrophages were washed with PBS and 3×10^6 cells were plated in 6-cm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) in 3 ml of EM. An antigen prepartion of 3LL tumor cells was added to some of the suspensions before plating in an equivalent amount of 2×10^6 tumor cells per 10^6 macrophages. Three to 4 hr later, the medium was replaced by 4 ml of EM + 10% fetal calf serum (FCS) (Reheis Chemical Co., Phoenix, Ariz.). By this time most of the cells were attached to the plate in a monolayer form. All the attached cells were able to phagocytize India Ink particles when incubated for 1 hr with suspensions of 20% India Ink in PBS. On the next day, the medium was replaced by suspensions of 40×10^6 SC from C57BL mice in 4 ml of EM + 15% FCS and incubated with the macrophage monolayers for 4 days. One milliliter of EM + 25% FCS was added on the 1st and 3rd days of incubation. On the 4th day, the lymphocytes were collected from the macrophage monolayer by gently pipetting. In order to remove residual macrophages from the sensitized lymphocytes we treated the cells collected by the following procedures: The cells were suspended in EM + 5% FCS at a concentration of 5×10^6/ml and 12 ml of these suspensions were incubated in 10-cm plastic Petri dishes (Falcon Plastics) for 1 hr. After this period the nonadherent cells were collected by gently pipetting and introduced into nylon wool columns for 1 hr as described (23). This method was shown to remove B cells and macrophages (23). We tested the eluted cells for the presence of B lymphocytes by incubating for 1 hr in ice 10^6 cells in 1 ml of fluorescein-conjugated goat anti-mouse γ-globulin (Hyland, Costa Mesa, Calif.) diluted 1:40 with PBS. All the cells were found negative. Using this method, we found 30 to 40% positive cells in normal SC from C57BL mice. Hence, the eluted cells did not contain B lymphocytes. The cells eluted from the column were washed with PBS and tested for cytotoxic activity *in vitro* or for their activity against tumor cells *in vivo*.

B) Direct sensitization on fibroblasts or tumor cell monolayers. Monolayers of tumor cells or fibroblasts were prepared and treated with mitomycin C as described (6). 40×10^6 SC from C57BL mice were incubated with the sensitizing monolayers in 4 ml of EM + 15% FCS in 6-cm plastic Petri dishes (Falcon). One milliliter of EM + 25% FCS was added on the 1st and 3rd days of incubation. On the 4th day the SC were collected from the monolayers by gentle pipetting. The SC were separated from contaminating dead tumor cells by centrifugation in FCS for 4 min at $6.5 \times$ G as described (7). The SC were separated from B cells and macrophages as above, washed with PBS, and tested for cytotoxic activity *in vitro*.

In vitro cytotoxicity assay. The induction of sensitization and the development of effector lymphocytes were tested by the assay of end labeling of the remaining target cells with ^{3}H-leucine. The assay was described in detail (6). Briefly, fibroblasts or tumor cells were plated 10^5 cells/well into microtiter plates (Greiner, Nurtingen, Germany). Twenty-four to 48 hr later the medium and the nonadherent cells were removed and 10^6 SC suspensions in EM + 15% FCS were added to the wells and incubated for 22 hr. At the end of the incubation the SC and non-attached (dead) cells were removed and the remaining target cells were given a 1 to 2 hr pulse of 1 μCi ^{3}H-leucine (specific activity 1 Ci/mmole) (The Radiochemical Center, Amersham, England). The cells were precipitated and washed in 5% trichloroacetic acid, dissolved in 1 N NaOH and counted in toluene scintillation fluid. All experiments were done in three to five replicates. Percentage of cytotoxicity was calculated as follows:

$$\left[1 - \frac{\text{CPM in test group}}{\text{CPM in control group}}\right] \times (-100)$$

With this calculation, each cytotoxicity value is expressed by a negative number (minus sign). Plus (+) values represent a greater number of CPM in the experimental group compared to the control group. The mechanism of this apparent enhancement is unknown. The control group was chosen according to the type of sensitization. The control for sensitization by macrophages was lymphocytes sensitized on macrophages without antigen and the control was lymphocytes sensitized on syngeneic fibroblasts for sensitization on tumor cell monolayers. We found that such controls are better than medium control since the presence of lymphocytes on the target cells sometimes caused nonspecific damage to the target cells. All the tissue culture procedures and the sensitization *in vitro* were performed in 37°C humidified air with 10% CO_2. Viable cell counting was done by the exclusion of 0.1% trypan blue.

In vivo assay for tumor development. Suspensions of sensitized lymphocytes were mixed together with tumor cells in 0.05 ml EM and were injected subcutaneously into the right footpads of syngeneic mice with a No. 27 needle. Each experimental group contained 10 mice. The mice were examined daily for tumors. The development of a visible tumor, as compared to the nontreated left footpad, was considered significant. The cumulative percentage of mice developing tumors is shown in the figure, as well as the percentage of mice that did not develop tumor at all.

RESULTS

We found in earlier studies that fibroblasts (1) and tumor cell monolayers (4, 7) were able to sensitize lymphocytes *in vitro*. The immunogens involved in this sensitization were cell-bound membrane antigens. We first tested whether syngeneic macrophages which had been fed with tumor cell antigen preparation could replace intact tumor cells as effective immunogenic agents for primary induction of lymphocyte sensitization *in vitro*.

C57BL spleen cells were incubated for 4 days either with 3LL tumor cell monolayers, or with C57BL macrophages that had been fed with 3LL antigen preparation. After this period, the lymphocytes were collected, separated from B cells and residual macrophages, and tested for their cytotoxic activity against allogeneic fibroblasts or allogeneic and syngeneic tumor cells (Table I).

The results indicated that both procedures of sensitization were effective. The sensitized T lymphocytes manifested specific cytotoxic activity and the degree of immunospecificity was similar in both procedures. In each experiment, high cytotoxic values were obtained on the specific 3LL target but also on the syngeneic L tumor, probably due to cross antigenicity between the tumors. Much smaller values of cytotoxicity were obtained on the allogeneic fibroblasts and no effect or enhancement of target cell growth was observed by the allogeneic KHT tumor.

What is the time required for sensitization of lymphocytes by macrophages? We found before that during 6 to 18 hr of lymphocyte sensitization *in vitro*, initiator T lymphocytes developed (24, 25) but cytotoxic lymphocytes appeared only after 3 days of direct sensitization *in vitro* (6) or after the

recruitment of effector lymphocytes *in vivo* by the initiator cells (25).

We therefore investigated whether cytotoxic lymphocytes could be obtained by less than 4 days of interaction with the sensitizing macrophages. C57BL spleen cells were incubated with antigen-fed macrophages for 2 and 4 days and their cytotoxic activity against 3LL cells or fibroblasts was measured (Table II).

The results indicated that cytotoxic capacity was obtained after 4 days, but not after 2 days of sensitization. The highest cytotoxic activity was against 3LL cells. Hence the generation of cytotoxic lymphocytes by antigen-fed macrophages requires 4 days and shorter interaction period was not sufficient.

Is the capacity to induce specific sensitization a unique property of macrophages, or can other types of adherent non-lymphoid cells, present extrinsic antigens in an immunogenic form?

We tested this question by comparing syngeneic fibroblasts and macrophages, which were treated with antigen preparation in the same manner, for their potential to induce sensitization against 3LL cells (Table III). The results showed that the inductive potential was limited to macrophages and was not obtained by using fibroblasts. Hence, macrophages possess the ability to process the antigen and/or to expose it in an immunogenic form to lymphocytes.

In the above experiments the interaction *in vitro* was performed between antigen-fed macrophages and syngeneic lymphocytes. Can such an interaction occur between histoincompatible populations of macrophages and lymphocytes? We investigated this possibility by using either C3H or C57BL antigen-fed macrophages to induce sensitization in C57BL lymphocytes. In this experiment we also tested the possibility that the preparation of 3LL antigen by itself was able to sensitize lymphocytes directly, without preincubating it with macrophages (Table IV). The results indicated that only syngeneic macrophages were able to induce sensitization of lymphocytes. The allogeneic combination did not produce cytotoxic lymphocytes. The antigen preparation by itself was also non-immunogenic under the conditions of the experiments.

What is the influence of lymphocytes sensitized by macrophages on tumor development *in vivo?* The improtance of this question was emphasized by our earlier finding that sensitized lymphocytes could produce opposite activities *in vivo* and *in vitro*. Lymphocytes, which were sensitized directly against syngeneic tumor cells *in vitro*, manifested cytotoxic activity *in vitro* but enhanced tumor growth when injected together with tumor cells into syngeneic recipients (4).

C57BL spleen cells, which had been sensitized by antigen-fed macrophages, were mixed together with 3LL cells and

TABLE I

Cytotoxic activity of lymphocytes sensitized by antigen-fed macrophages or by tumor cells

Sensitization	Target Cells							
	C3H-fibroblast		3LL		L		KHT	
	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity
	cpm ± *S.D.*	%	*cpm* ± *S.D.*	%	*cpm* ± *S.D.*	%	*cpm* ± *S.D.*	%
Macrophages								
Medium control	45145 ± 8606		15346 ± 3098		91806 ± 4759		4286 ± 334	
Macrophages	29889 ± 3399		12248 ± 988		54805 ± 2025		16508 ± 1507	
Macrophages + antigen[a]	25703 ± 1273	−14	6945 ± 439	−57	32011 ± 6085	−40	16437 ± 62	−1
Intact cell monolayers								
Medium control	202491 ± 35146		153578 ± 11082		257861 ± 47425		54397 ± 2664	
C57BL fibroblasts	177299 ± 13362		120248 ± 12677		243098 ± 15273		69680 ± 2756	
3LL[b]	146855 ± 1533	−17	64774 ± 11293	−46	208891 ± 15284	−14	100105 ± 9129	+44

[a] The control for this group was sensitization by nontreated macrophages.

[b] The control for these groups was sensitization by C57BL fibroblasts.

TABLE II

Cytotoxic activity of lymphocytes snesitized by antigen-fed macrophages for 2 or 4 days[a]

Sensitization		Target Cells					
		C3H fibroblasts		C57BL-fibroblasts		3LL	
Macrophages	Days	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity
		cpm ± *S.D.*	%	*cpm* ± *S.D.*	%	*cpm* ± *S.D.*	%
Medium control		100256 ± 10027		97970 ± 8195		38628 ± 5087	
Macrophages	2	84978 ± 6162		52916 ± 19909		24118 ± 6402	
Macrophages + antigen		94248 ± 6474	+11	65029 ± 5939	+23	22064 ± 6638	−9
Medium control		56327 ± 6133		36936 ± 3214		11406 ± 2629	
Macrophages	4	45714 ± 2218		50248 ± 2526		18794 ± 3553	
Macrophages + antigen		39531 ± 1415	−14	37047 ± 2995	−26	6790 ± 3496	−64

[a] C57BL SC were sensitized by C57BL antigen-fed or nontreated macrophages for 2 or 4 days.

TABLE III

Cytotoxic activity of lymphocytes sensitized by macrophages or fibroblasts fed with antigen[a]

Sensitization	Target Cells			
	3LL		C57BL fibroblasts	
	Incorporation	Cytotoxicity	Incorporation	Cytotoxicity
	cpm ± *S.D.*	%	*cpm* ± *S.D.*	%
Medium control	522317 ± 52564		655989 ± 170575	
C57BL fibroblasts	535543 ± 40161		247411 ± 46558	
C57BL fibroblasts + antigen	508404 ± 37478	−5	312656 ± 72437	+26
Macrophages	503342 ± 36220		300246 ± 40588	
Macrophages + antigen	115898 ± 23491	−77	269161 ± 28689	−10

[a] C57BL SC were sensitized by antigen-fed C57BL macrophages or by C57BL fibroblasts which were exposed to the same antigen. The controls were nontreated C57BL macrophages or fibroblasts.

TABLE IV

Cytotoxic activity of lymphocytes sensitized by syngeneic or allogeneic antigen-fed macrophages[a]

Sensitization	Target Cells	
	Incorporation	Cytotoxicity
	cpm ± *S.D.*	%
Medium control	118331 ± 15028	
C3H macrophages	118869 ± 14918	
C3H macrophages + antigen	108268 ± 11592	−9
C57BL macrophages	144099 ± 21661	
C57BL macrophages + antigen	65800 ± 7566	−54
No macrophages + antigen	148286 ± 35766	+30

[a] C57BL SC were sensitized by C3H or C57BL antigen-fed macrophages. The controls were C3H or C57BL nontreated macrophages, respectively.

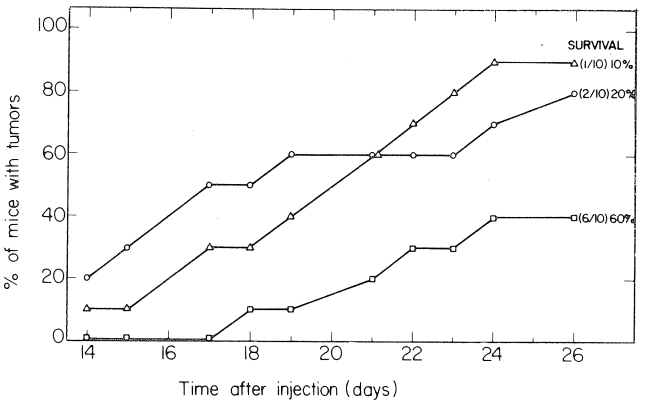

Figure 1. The *in vivo* activity of lymphocytes which were sensitized *in vitro* by antigen-fed macrophages. C57BL SC were sensitized by C57BL untreated (*circles*) or antigen-fed (*squares*) macrophages. Sensitized SC, 2×10^6, were mixed with 2×10^4 3LL cells in 0.05 ml EM and injected subcutaneously into the right food pads of C57BL mice. One group was injected with 2×10^4 3LL cells in 0.05 ml EM (*triangles*). The mice were observed for 3 months.

injected into syngeneic recipients (Fig. 1). We observed that lymphocytes sensitized by antigen-fed macrophages were not only cytotoxic *in vitro*, but had also protective activity *in vivo*. Such lymphocytes inhibited tumor development and reduced the percentage of tumor takes as compared with control lymphocytes or with tumor cells which were injected without lymphocytes.

DISCUSSION

Peritoneal macrophages, which had been fed with cell-free supernatants of 3LL tumor cells, served for sensitization of lymphocytes *in vitro*. Such treated macrophages were able to induce primary sensitization in syngeneic spleen cells against the tumor. The sensitized T lymphocytes had specific cytotoxic activity *in vitro* and protective activity against tumor development *in vivo*. These activities were always higher when the lymphocytes were sensitized by antigen-fed macrophages as compared to nontreated macrophages.

In order to remove residual macrophages that were collected with the sensitized lymphocytes, the cells were routinely separated after the sensitization by adherence to a plastic surface and by incubation on nylon wool columns, and only the eluted population of T lymphocytes was tested.

During the incubation of the lymphocytes *in vitro*, a process of sensitization and accumulation of effector cells occurred. As we observed before, the optimal period for direct sensitization by fibroblasts or tumor cells was 4 to 5 days (1, 6). Exposure of the lymphocytes to antigen-fed macrophages for only 2 days was not sufficient and 4 days of incubation were needed in order to obtain cytotoxic lymphocytes. The interaction of the antigen with the macrophages seems to be essential for lymphocyte sensitization. The antigen preparation by itself was not immunogenic to SC in this system although SC contain macrophages. It is possible that the quantity or quality of spleen macrophages was not adequate for *in vitro* sensitization and preincubation of the antigen with peritoneal macrophages was required. Syngeneic fibroblasts, which had been exposed to antigen preparation in the same way as the macrophages, were also non-immunogenic to sc. These results suggested that the sensitization of the lymphocytes was directed toward antigens which were exposed in an immunogenic form by the macrophages.

The physiologic nature of the interaction between the macrophages and the lymphocytes was supported by the finding that only in syngeneic, but not in allogeneic combination, did the sensitization occur.

A requirement for histocompatibility was also found for the priming of helper activity of T lymphocytes by soluble antigen-treated macrophages in antibody production by B cells (26), and also for the proliferative reaction of primed T lymphocytes mediated by antigen-fed macrophages (27). It is not clear, however, if the genetic matching between macrophages and T lymphocytes was required to avoid allosensitization of the lymphocytes or in order to enable suitable contact for the sensitization.

The effector phase in the present experiments was mediated by T lymphocytes since we removed B cells and macrophages

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before the tests. We have also tested the macrophages for cytotoxic activity and found that macrophages, taken from thioglycollate-treated mice, destroyed tumor cells and fibroblast monolayers. This nonspecific cytotoxicity was not increased if the macrophages were fed with tumor antigen (unpublished results). Hence, undetected small numbers of macrophages which might have contaminated the population of sensitized T lymphocytes, could not be responsible for the specific cytotoxicity observed.

Cytotoxic activity mediated by macrophages from immunized animals was found by others to be either specific (16) or nonspecific (15, 16). It was also suggested that an arming factor, secreted by activated T lymphocytes, triggers macrophages to become cytotoxic (28). These findings attribute to macrophages an effector role in cell-mediated immunity.

In addition, macrophages contribute to the proliferative activity of T lymphocytes. It was found that sheep erythrocytes, which were processed by macrophages, elicited a stronger delayed hypersensitivity response than the natural antigen (29). Macrophages also were found to be essential for the triggering of T lymphocyte proliferation by BCG (13) and to secrete lymphocyte activating factors which promoted lymphocyte proliferation (12). These findings as well as our observations, indicate that the macrophage participate in the afferent phase of cell-mediated immunity.

Hence, the development of cytotoxic cells can process in at least two directions: 1) Antigen-primed macrophages activate cytotoxic T lymphocytes. 2) Antigen-primed T lymphocytes activate cytotoxic macrophages. It is very well possible that both reactions occur *in vivo* and augment each other. Bi-directional interaction between T lymphocytes and macrophages was also suggested from the analysis of the mode of action of BCG (13).

In vitro sensitization of lymphocytes against tumor cells has been proposed as a useful approach for circumventing blocking activity that might prevent the mounting of an efficient protective immune response *in vivo* (30). However, direct sensitization of lymphocytes against tumor cells did not always produce protective effector cells. Lymphocytes sensitized against syngeneic tumor cells *in vitro* had cytotoxic activity *in vitro*, but enhanced tumor growth when injected together with tumor cells into syngeneic recipients (4). But in addition *in vitro* sensitized lymphocytes had protective activity against metastatic development (7) or local tumor growth (31) when injected systemically without direct contact with the tumor cells.

The sensitization of lymphocytes by antigen-fed macrophages seems to be more efficient for obtaining protective activity than direct sensitization against tumor cells. The lymphocytes sensitized by macrophages, inhibited local tumor growth even if injected together with tumor cells. It is not clear yet what is the difference between the effector cells obtained by these two procedures.

The use of macrophages as sensitizing agents against tumor cells could also overcome methodologic difficulties which were found in the direct sensitization against the tumor *in vitro* (7). 1) Viable tumor cells are not present in the system and thus are not hazardous if the sensitized lymphocytes would have to be injected *in vivo*. 2) The system enables the sensitization of lymphocytes *in vitro* against tumors which do not produce a stable monolayer. 3) The sensitization by macrophages might increase the immunogenicity of intact tumor cells.

The model presented here, has the above mentioned advantages for *in vitro* sensitization of lymphocytes against tumor cells. It produces *in vitro* a population of protective and cytotoxic effector lymphocytes and also enables the investigation of the interaction between macrophages and lymphocytes in the immune sensitization against cell bound antigens.

The mechanism of T lymphocyte sensitization, the genetic requirements for the interaction between these two populations of cells, and nature of the cell-bound antigens are some additional relevant questions amenable for investigation by this system.

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