Immune reactivity in SL2 lymphoma-bearing mice compared with SL2-immunized mice

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Summary. We have studied the rather paradoxical phenomenon of the growth of an antigenic tumor in an immunocomponent host. This phenomenon was studied by comparing (a) the lymphocyte reactivity and (b) the macrophage cytotoxicity, during SL2 growth in DBA/2 mice (SL2-bearing mice) and in DBA/2 mice immunized against SL2 tumor cells (SL2-immune mice). Immune mice rejected a challenge of tumor cells. The immune T-lymphocytes rendered macrophages cytotoxic (arming) and were able to transfer tumor resistance to naive animals. Nonimmunized mice did not reject a challenge of SL2 cells. In these tumor-bearing mice various forms of immune reactivity were tested. Lymphocytes with the capacity to arm macrophages could not be found in the lymphoid organs. However, lymphocytes isolated from the tissue directly surrounding the subcutaneous SL2 tumor could arm macrophages in vitro.

Shortly after subcutaneous tumor grafting cytotoxic macrophages were found in the peritoneal cavity. In the serum macrophage arming factors were detected that rendered macrophages cytotoxic in vitro. This cytotoxicity of the peritoneal macrophages and the presence of macrophage arming factors in the serum showed a similar biphasic pattern. The first phase of cytotoxicity between day 3 and 8 after tumor grafting was tumor (SL2) specific. The second phase from day 12 and onwards was not tumor specific. During the first 4 days after SL2 grafting the DBA/2 mice expressed a specific concomitant immunity to a second tumor graft. Then 7 or more days after grafting the first SL2 tumor, the concomitant immunity was nonspecific as the growth of a second SL2 tumor graft and a L5178Y (DBA/2) tumor graft were inhibited. In addition, the immune suppressive activity of serum and lymphocytes was tested. Neither serum nor lymphocytes from SL2-bearing mice suppressed the macrophage arming capacity of SL2 immune lymphocytes. Lymphocytes from tumor-bearing mice did not inhibit the capacity of SL2-immune lymphocytes to transfer resistance to naive animals. On the contrary, lymphocytes obtained from SL2-bearing mice 14 days after SL2 grafting transfered tumor resistance in a Winn-type assay. These data suggest that the growth of an antigenic tumor is due to the inability of the immune system to mount an effective antitumor effector cell population during tumor growth, rather than an immune suppression of the antitumor reactivity, as a limited immune reactivity could be detected in tumor-bearing mice, whereas immune suppression could not be detected.

Introduction

We have previously described the SL2 lymphosarcoma as a rapidly metastasizing T-cell tumor, that can kill DBA/2 mice after transplantation of 10^4 cells within 30 days [10]. Still, this tumor is antigenic as injection of irradiated SL2 cells leads to an effective immunization of DBA/2 mice. T-lymphocytes obtained from these immunized animals can transfer tumor resistance to tumor-bearing mice [27, 29] and induce specifically cytotoxic macrophages in vitro [7]. This indicated that the SL2 cells can be recognized immunologically by the DBA/2 mouse.

In the SL2-DBA/2 murine tumor system [27, 28] and in other murine tumor systems [2, 22] the resistance against the tumor has been shown to be dependent on cooperation between T-lymphocytes and macrophages. The T-lymphocytes are important for the immunological recognition of the tumor cells, whereas, the macrophages probably, after activation by the lymphocytes, act as the major tumoricidal cell [5].

In this paper we have studied the reactivity of lymphocytes from SL2 tumor-bearing mice, to find out why the DBA/2 mouse cannot resist the growth of the antigenic SL2 tumor. In addition, we have studied the presence of cytotoxic macrophages and macrophage arming factors during tumor growth. To account for the low lymphocyte reactivity during tumor growth it was investigated whether the antitumor reactivity was inhibited by suppressor cells or suppressor factors in the serum, as has been described in other murine tumor systems [17, 18, 25, 26]. We also examined the alternative possibility, namely that the tumor-bearing animals are not able to mount an effective antitumor effector cell population. We have approached these questions by studying the ineffective T-cell reactivity and macrophage cytotoxicity in tumor-bearing mice in comparison with the effective T-cell reactivity and macrophage cytotoxicity in immunized mice.

The data obtained in the SL2 tumor-bearing DBA/2 mouse system favor an immune reactivity against the SL2 cells that does not lead to an effective effector cell population against the tumor, rather than suppression of the antitumor immune response.

Materials and methods

Animals

Inbred DBA/2 and BALB/c mice (6–10 weeks) were obtained from CPB-TNO, Zeist, The Netherlands. Inbred C57BL/10ScCR mice (6–10 weeks) were purchased from Bomholtgård.

Tumors

The DBA/2 tumors used were the SL2 lymphoma, which arose spontaneously and the chemically induced lymphoma L5178Y. The BALB/c tumor used was the plasmacytoma MPC11. The C57BL tumor was the EL4 lymphoma which was chemically induced. All tumors grew as ascitic tumors in the peritoneal cavity. The tumors were maintained by weekly i.p. passage. In vitro SL2, L5178Y, and EL4 had a mean generation time of 16–20 h; the mean generation time of MPC11 was 24–32 h. The tumors were free of Mycoplasma and cross-reacting viruses.

Tumor immunization and tumor grafting

DBA/2 mice were immunized by 2 injections of 10^7 irradiated (5,000 rad) SL2 cells i.p. on days −20 and −10. After this immunization 95% of the mice could resist a lethal dose of 5×10^6 nonirradiated SL2 cells injected i.p. on day 0. Peritoneal lymphocytes used to arm macrophages were harvested on day 7 (after injection of non irradiated cells) [7]. Spleen lymphocytes used for transfer experiments were harvested on day 12 [27]. To obtain tumor-bearing animals, DBA/2 mice were injected s.c. with 10^4 SL2 cells in the flank.

To test concomitant immunity, SL2-bearing DBA/2 mice were injected with 10^4 tumor cells in the groin. At this site tumors developed as ellipsoids and the tumor mass was calculated using the formula $4/3\ \Pi A^2B$, where A is the length of the short axis and B is the length of the long axis of the tumor mass, as measured with calipers.

Histology and cytology

Subcutaneous tumors and the skin covering the tumor were removed, fixed in 4% formaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin eosin. Cytocentrifuge preparations were made from cell suspensions to determine the cell composition. The macrophage content in cell suspensions was assayed with nonspecific esterase using α-naphthyl acetate as substrate [30].

Cell cultures

A. *Tumor cell cultures.* Tumor cells were harvested from the peritoneal cavity of mice 7–11 days after transplantation, washed with Fischer's medium and suspended at a concentration of $1.5–2 \times 10^5$ cells/ml growth medium (Fischer's medium supplemented with 10% fetal bovine serum (FBS)).

B. *Lymphocyte cultures.* Lymph nodes or spleens were squeezed through a metal sieve in Fischer's medium. The cell suspension was centrifuged and the cell pellet resuspended. After removal of dead cells and cell debris by glass wool filtration the lymphocytes were resuspended in growth medium (2×10^6 cells/ml). The suspension was cul-

tured for 2 h at 37 °C in glass flasks before use, to remove most of the adherent cells.

Peritoneal lymphocytes were obtained from peritoneal (exudate) cells. The macrophages were removed from the suspension by glass adherence (culturing twice for 1 h at 37 °C in glass flasks). After this procedure the cell suspensions contained less than 3% nonspecific esterase positive macrophages. The lymphocytes were suspended in growth medium (2×10^6 cells/ml).

Lymphocytes surrounding the subcutaneous SL2 tumor were isolated from the skin. Between day 3 and 10 after tumor grafting the skin above and about 3 mm around the tumor (injection site) was shaved and excised. Tumors were 0.5–6 mm in diameter at that time and only loosely attached to the skin, so the skin could easily be removed from the tumor. After rinsing in Fischer's medium, the skins of 10 mice were cut in 2 mm^2 pieces and incubated for 60 min at 4 °C in 10 ml Fischer's medium containing 10% FBS and 0.2% collagenase. Subsequently, this suspension was incubated for 75 min at 37 °C with constant stirring. The cell suspension was filtered over glass wool to remove cell debris and washed twice in Fisher's medium to remove the collagenase. To remove the adherent cells (macrophages and fibroblasts) from the cell suspension the cells were incubated for 1 h at 37 °C in a glass flask. The final cell suspension was used at a cell concentration of 2×10^6 lymphocytes/ml. Only lymphocyte suspensions containing less than 5% tumor cells were used.

C. *Macrophage monolayers.* Peritoneal cell suspensions containing 8×10^5 or 4×10^5 macrophages were seeded into 24 or 48-well culture dishes respectively (Costar, $\varnothing$ 16 mm or 11.3 mm). The macrophages were allowed to adhere at 37 °C. After 1–2 h of incubation the cultures were washed carefully with jets of medium from a pipet to remove nonadhering cells. The adhering cells formed a confluent monolayer as observed by phase contrast microscopy. At least 95% of the cells were characterized as macrophages as described previously [21]. The peritoneal macrophages from normal mice used did not display natural cytotoxicity against tumor cells [20].

Direct arming of macrophages by lymphocytes

Macrophages were rendered specifically cytotoxic by direct arming with lymphocytes as described previously [7, 8]. Briefly: unless stated otherwise, macrophage monolayers were incubated with lymphocytes (either from immune animals or tumor-bearing animals) and SL2 cells at a macrophage:lymphocyte:tumor cell ratio of 10:20:1 for 24 h at 37 °C (arming). After incubation, the lymphocytes and tumor cells were washed from the macrophage monolayer and the macrophages were challenged with a suspension of 10^5 SL2 tumor cells/ml (macrophage:tumor cell ratio of 10:1).

Macrophages from SL2-immunized mice and SL2-bearing mice

DBA/2 mice immunized i.p. with 10^7 irradiated (5,000 rad) SL2 cells on days −20 and −10, received an additional injection on day 0 of 5×10^6 nonirradiated cells. On different days after the last injection, peritoneal cells were collected. Peritoneal cells from tumor-bearing mice were harvested on different days after s.c. tumor injection. The

peritoneal cells were seeded in flat bottomed culture dishes. The macrophages were allowed to adhere for 2 h at 37 °C. Subsequently the macrophage monolayers were washed to remove nonadherent cells and the cytotoxicity was determined. As control, peritoneal macrophages from normal mice were used.

Collection of serum

Serum from normal and tumor-bearing animals was obtained by puncture of the retro orbital plexus with a fine glass pipet. The sera were used immediately or stored at −20 °C.

Macrophage cytotoxicity induced by serum

Serum from tumor-bearing and normal mice was collected. Monolayers from normal peritoneal macrophages were incubated for 4 h with serum or a serum dilution from tumor-bearing mice. Subsequently, the monolayers were washed and the cytotoxicity determined. As control, peritoneal macrophages were incubated with serum from normal mice.

Cytotoxicity

Cytotoxicity was assessed after 24 h comparing the growth of tumor cells in the test system with the growth of tumor cells in the control [9]. In control experiments for armed macrophages, normal macrophages incubated with normal lymphocytes and SL2 cells were used. The growth of tumor cells on control macrophages was no different from the growth of SL2 cells only or the control macrophages were slightly growth stimulating (less than 10%). Before counting, the cultures were incubated with 10 μl Indian ink (1/10 diluted) for 30 min. Macrophages phagocytose Indian ink in contrast to tumor cells. Only viable tumor cells were counted as judged by trypan blue exclusion. Cytotoxicity was expressed as: $CI = (1-T/N) \times 100$, where CI is the cytotoxicity index, N is the number of tumor cells in controls, and T is the number of cells in the test system.

Results

SL2-Immune DBA/2 Mice

Macrophage arming lymphocytes in SL2-immunized DBA/2 mice. DBA/2 mice can be immunized against SL2 cells. These mice resisted a challenge of 5×10^6 nonirradiated SL2 cells. This indicated that DBA/2 mice recognized the SL2 tumor cells as "foreign". Lymphocytes obtained from these mice rendered macrophages specifically cytotoxic (Table 1). The capacity of lymphocytes to render macrophages cytotoxic was abrogated by treatment of the lymphocytes with anti-Thy 1 serum and complement but not with anti-murine Ig (K+λ) serum and complement. Treatment of monolayers of armed macrophages with anti-Thy 1 or anti-murine Ig sera and complement did not alter the cytotoxicity (data not shown [7]). This indicated that the cytotoxicity was induced by T-cells, but that the cytotoxicity itself was not due to T-cells and not due to antibody dependent cell mediated cytotoxicity but expressed by the armed macrophages. The specificity for SL2 cells was not due to a higher sensitivity of SL2 cells for macrophage cytotoxicity, as in other systems (allogeneic and syngeneic) other target specificity spectra were shown [7].

Table 1. Specific cytotoxicity of macrophages armed by SL2-immune lymphocytes[a]

Target cell	Strain of origin	H-2 type	Cytotoxicity index[b]
SL2	DBA/2	d	60 ± 7
L5178Y	DBA/2	d	27 ± 9[c]
MPC11	BALB/c	d	20 ± 5[c]
EL4	C57BL	b	19 ± 3[c]

[a] DBA/2 mice were immunized i.p. with 10^7 irradiated SL2 cells on days −20 and −10. On day 0 the mice received an i.p. injection of 5×10^6 nonirradiated SL2 cells. On day 7 the peritoneal lymphocytes were harvested. These SL2-immune lymphocytes were incubated with a monolayer of normal macrophages and SL2 cells. After 24 h the lymphocytes and SL2 cells were removed and the cytotoxicity of the armed macrophages was determined against various target cells [7]

[b] Mean ± SEM of 4 experiments performed in triplicate

[c] Cytotoxicity significantly different from the cytotoxicity against SL2 cells ($P < 0.05$)

Macrophage cytotoxicity in SL2-immunized DBA/2 mice

DBA/2 mice were immunized i.p. with 10^7 irradiated SL2 cells on days −20 and −10. Peritoneal macrophages harvested on day 0 were not cytotoxic. When immunized mice were injected i.p. on day 0 with 5×10^6 SL2 cells, these cells were rejected within 3–4 days. Peritoneal macrophages harvested from these mice on day 3 expressed a high cytotoxicity. This macrophage cytotoxicity slowly decreased thereafter and the cytotoxicity disappeared between days 10 and 15 (Fig. 1). The specificity of the cytotoxicity of these immune peritoneal macrophages is shown in Table 2. On day 4 the cytotoxicity of the peritoneal macrophages was nonspecific, that is the cytotoxicity against SL2 cells was not significantly higher than against other tumor target cells. The cytotoxicity of the macrophages harvested on day 7 was, however, specific as the cytotoxicity against SL2 cells was at least twice as high as the cytotoxicity against other tumor cells. The cytotoxicity was not due to T-cells as treatment of the macrophage monolayers with anti-Thy 1 serum and complement did not reduce the cytotoxicity (data not shown).
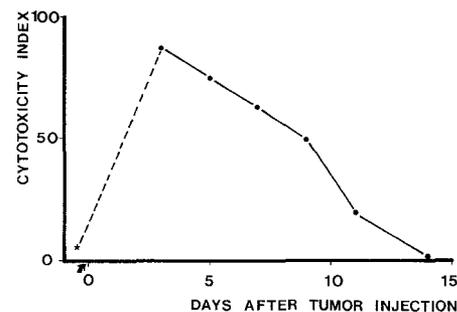

Fig. 1. Cytotoxicity of peritoneal macrophages in mice immunized against SL2 cells after SL2 cell challenge. DBA/2 mice were immunized i.p. with irradiated SL2 cells on days −20 and −10. On day 0 there was no macrophage cytotoxicity (*). On day 0 SL2 cells were injected i.p. (↗) and different days after this injection peritoneal macrophages were harvested and tested for their cytotoxicity. Mean of a representative experiment

Table 2. Specificity of the cytotoxicity of peritoneal macrophages from mice immunized with SL2 cells[a]

Target cell	Strain origin	H-2 type	Cytotoxicity index[b]	
			Day 4[c]	Day 7[d]
SL2	DBA/2	d	69 ± 8	46 ± 4
L5178Y	DBA/2	d	64 ± 5	12 ± 10[e]
MPC11	BALB/c	d	59 ± 8	18 ± 8[e]
EL4	C57BL	b	62 ± 4	14 ± 7[e]

[a] DBA/2 mice were immunized i.p. with 10^7 irradiated SL2 cells on days -20 and -10. On day 0 the mice were injected with 5×10^6 SL2 cells. On day 4 or 7 the peritoneal cells were harvested and cultured for 2 h in flat bottomed wells. The nonadherent cells were washed off and the macrophage cytotoxicity was tested against various target cells

[b] Cytotoxicity was determined after 24 h. Normal peritoneal macrophages were used as controls

[c] Mean $\pm$ SEM of 2 experiments performed in triplicate

[d] Mean $\pm$ SEM of 5 experiments performed in triplicate

[e] Cytotoxicity significantly ($P < 0.05$) different from the cytotoxicity against SL2 cells

SL2-Bearing DBA/2 mice

Macrophage arming lymphocytes in SL2-bearing DBA/2 mice. To test whether lymphocytes from SL2-bearing animals (s.c. SL2 tumor in the flank) were able to induce macrophage cytotoxicity, lymphocytes were collected from draining lymph nodes, spleen, and peritoneal cavity on different days after tumor grafting. None of these lymphocytes showed a significant induction of macrophage cytotoxicity (data are partly given in Table 3).

It was then tested whether injection of a second SL2 tumor (10^4 cells/mouse) in the opposite flank could attract or induce reactive lymphocytes to the draining lymph nodes or the spleen. In one group, mice were injected with the first SL2 tumor graft on day 0. Part of this group received a second tumor in the opposite flank on day 4. On day 5 the lymph node lymphocytes draining the first and second SL2 tumor and the spleen lymphocytes were tested for their macrophage arming capacity. Both the draining lymph node lymphocytes from the first tumor and spleen lymphocytes showed a capacity to induce macrophage cytotoxicity, however, only when a second tumor was injected on day 4. Lymphocytes from the lymph node draining the second tumor graft were not able to arm macrophages (Table 3).

In a second group of DBA/2 mice the first tumor was injected on day 0. This group of mice was divided in three subgroups. The first subgroup received no second tumor. The second subgroup received a second tumor in the opposite flank on day 4, and the third subgroup received a second tumor on day 11. On day 12 the lymph node lymphocytes draining either the first or the second tumor or spleen lymphocytes from all three subgroups were tested for their macrophage arming capacity. Only when a second tumor graft was injected either on day 4 or day 11, did the lymph node lymphocytes draining the primary tumor show a low macrophage arming capacity. No macrophage arming activity was found in the draining lymph node of the second tumor. The spleen lymphocytes were only able to arm macrophages in vitro when the second tumor was injected 1 day before the lymphocytes were collected (Table 3). None of the lymphocytes were able to render macrophages cytotoxic if SL2 cells were absent during the arming of the macrophages. This indicated that the lymphocytes had to be triggered in vitro before they arm macrophages.

In summary, these data showed that no arming lymphocytes were present in the lymphoid organs of tumor-bearing mice. After injection of a second tumor graft, arming lymphocytes appeared in the draining lymph node of the first tumor, and for a short period of time in the spleen.

Localization of SL2-sensitized lymphocytes at the site of the tumor

In view of the above, we investigated whether macrophage arming lymphocytes were present in tumor-bearing animals in the tumor or in the tissue surrounding the tumor. It was possible to "strip" the skin together with a layer of the infiltrating cells surrounding the subcutaneous tumor (Fig. 2A and B). This piece of skin was treated with collagenase and the suspensions obtained contained mainly lymphocytes and macrophages, and some fibroblasts and

Table 3. Macrophage arming capacity of lymphocytes in SL2-bearing DBA/2 mice[a]

Days between first graft and collection of lymphocytes	Days between first and second tumor graft	Cytotoxicity index of macrophages		
		Lymphocytes from lymph node draining first tumor	Lympocytes from lymph node draining second tumor	Spleen lymphocytes
5	–	11 ± 9	–	7 ± 4
5	4	18 ± 2[c]	9 ± 14	21 ± 5[c]
12	–	8 ± 9	–	9 ± 12
12	4	22 ± 5[c]	15 ± 10	6 ± 6
12	11	21 ± 4[c]	7 ± 2	21 ± 7[c]

[a] DBA/2 mice were injected on day 0 with 10^4 SL2 cells in the right flank. On day 4 or 11 some of the mice received a second tumor graft in the left flank. From these mice on day 5 or 12 lymph nodes draining the first or second tumor and spleen were collected. The macrophage arming capacity of the lymph node and spleen lymphocytes was tested (see Table 1). Cytotoxicity of armed macrophages was measured against SL2 cells

[b] Mean $\pm$ SEM of 3 experiments performed in triplicate

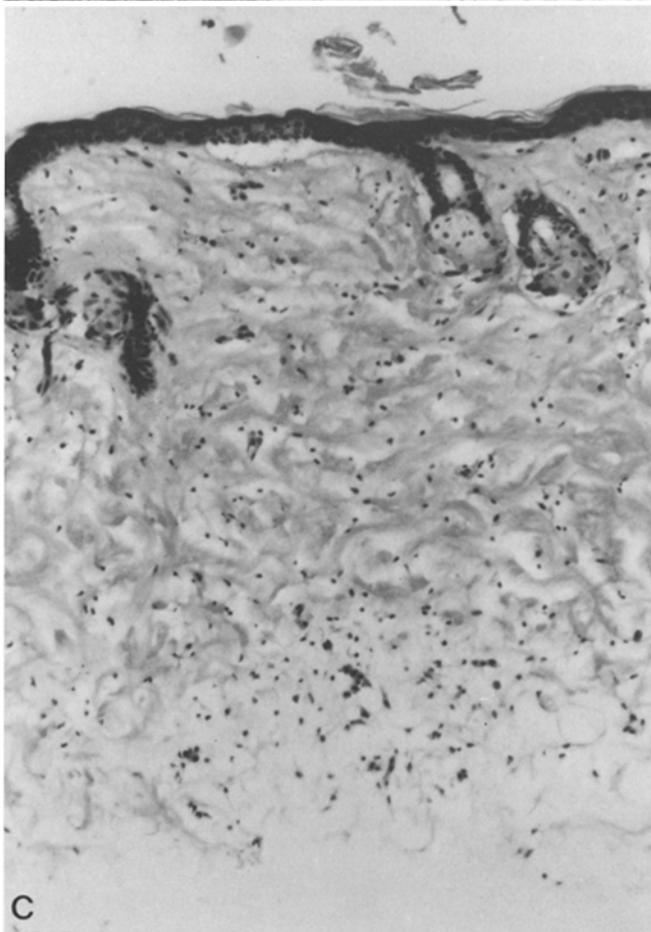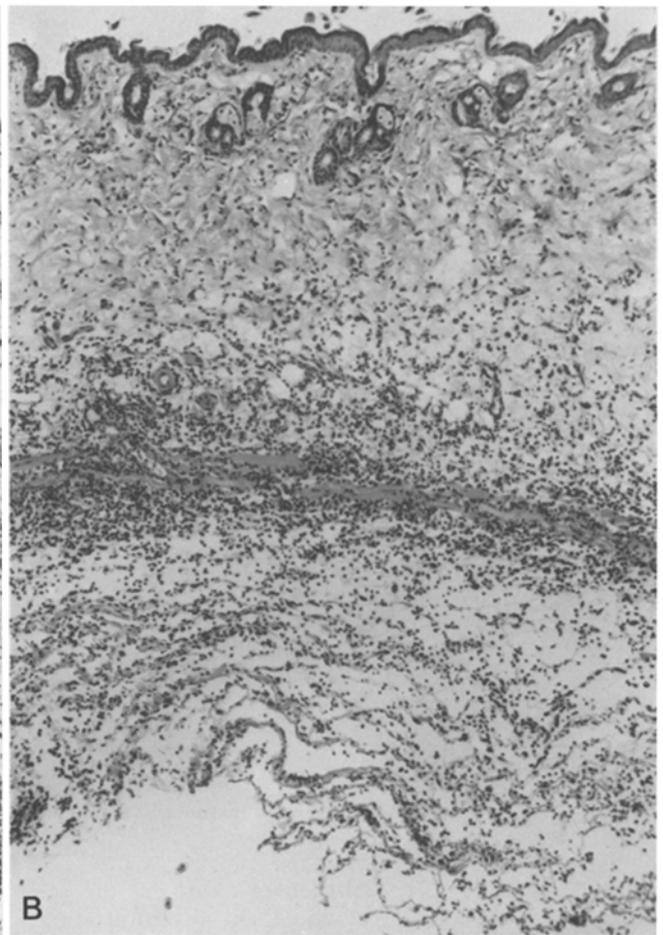[c] Cytotoxicity significantly different ($P < 0.05$) from control macrophages

Fig. 2. Histology of a s.c. SL2 tumor. **A:** Subcutaneous SL2 tumor 8 days after injection of 10^4 SL2 cells Tumor cells surrounded by infiltrating mononuclear cells (*) under the skin. Hematoxylin eosin staining ($\times 40$). **B:** Skin stripped from the tumor. Infiltrate of mononuclear cells was removed from the tumor together with the skin ($\times 60$). **C:** Skin stripped from the tumor after treatment with collagenase. Most of the infiltrating cells were removed by the collagenase treatment ($\times 100$)
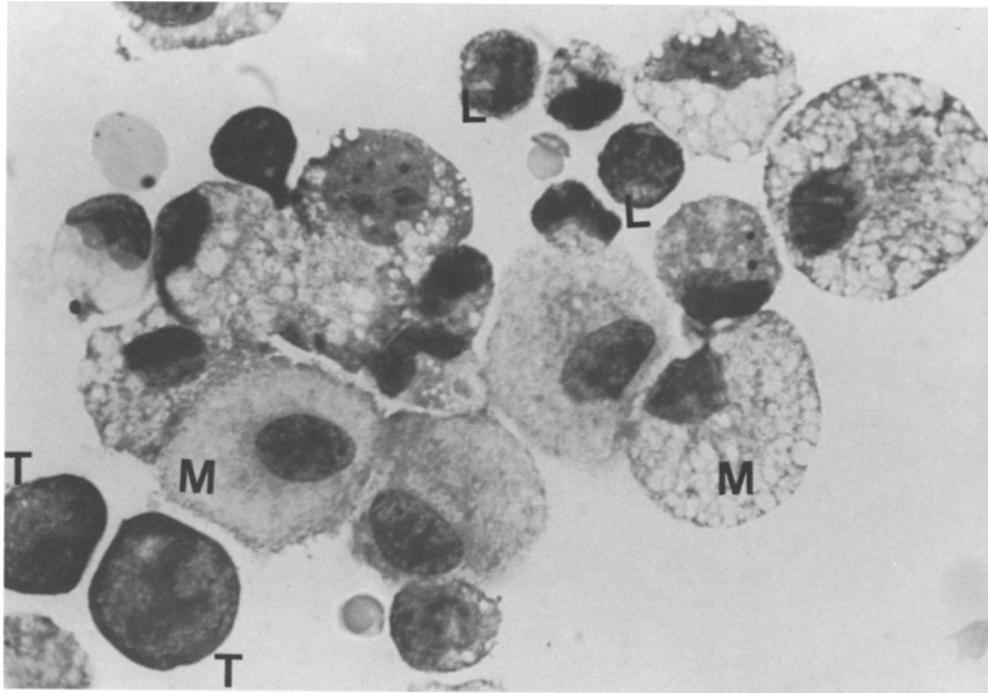
Fig. 3. Mononuclear cell infiltrate from a s.c. SL2 tumor. Cytospin preparation of mononuclear cells removed from the skin that was stripped from a subcutaneous tumor. The preparation contained macrophages (M), lymphocytes (L), and tumor cells (T) (× 800)

tumor cells (Fig. 3). After collagenase treatment the remaining pieces of skin hardly contained any infiltrating cells (Fig. 2C). These pieces of skin were removed from the cell suspension. This procedure could only be performed with relatively small tumors (between days 3 and 10). In suspensions of larger tumors (after day 10) too many tumor cells were present, as the tumors had infiltrated the skin by that time. These tumor cells could not be separated completely from the lymphocytes. The number of lymphocytes obtained from small tumors (days 3 to 5) was, however, small.

Most macrophages could be removed from the cell suspension by adherence. The remaining lymphocytes were tested for their macrophage arming capacity. As only small numbers of lymphocytes were obtained the macrophages were armed with relatively few lymphocytes. With SL2-immune lymphocytes routinely a macrophage:lymphocyte:tumor cell ratio of 10:20:1 was used to arm macrophages. In these experiments the ratio was between 10:12:1 and 10:1:1. As shown in Table 4 in most cases these relatively small numbers of lymphocytes induced significant macrophage cytotoxicity.

Macrophage cytotoxicity in SL2-bearing DBA/2 mice

To test the macrophage cytotoxicity in DBA/2 mice bearing a s. c. SL2 tumor, peritoneal macrophages were harvested on different days after s.c. injection of 10^4 SL2 cells. The macrophage cytotoxicity developed during tumor growth in a biphasic pattern (Fig. 4). Even on day 3 the peritoneal macrophages expressed a high cytotoxicity. This first phase of cytotoxicity was maximal on day 5 and decreased thereafter. Between days 8 and 10 no significant cytotoxicity was present. After day 10 the macrophage cytotoxicity increased again. Mice died or were killed between days 17 and 20.

To test the specificity of the macrophage cytotoxicity in tumor-bearing mice, macrophages were harvested on day 7 and day 14, and the cytotoxicity was tested (Fig. 5). The cytotoxicity of the macrophages harvested on day 7 was specific for the SL2 tumor cells. The cytotoxicity of the macrophages harvested on day 14 was nonspecific for

Table 4. Macrophage arming capacity of lymphocytes around a subcutaneous SL2 tumor[a]

Days between tumor grafting and collection of lymphocytes	Macrophage:lymphocyte:tumor cell ratio during arming[b]	Cytotoxicity index[c]
3	10:6:1	20[d]
5	10:3:1	24[d]
7	10:10:1	38[d]
7	10:10:1	47[d]
7	10:6:1	10
9	10:10:1	37[d]
10	10:12:1	6

[a] Lymphocytes removed from the subcutaneous tumor by collagenase treatment of the skin that was stripped from the tumor. Lymphocytes were harvested on different days after tumor injection (10^4 cells s.c.). These lymphocytes were tested for their macrophage arming capacity (see Table 1)

[b] In routine experiments lymphocytes were incubated during arming with macrophages and tumor cells at a macrophages:lymphocyte:tumor cell ratio of 10:20:1 (Table 1). Often insufficient lymphocytes were obtained from the pieces of skin and for this reason different numbers of lymphocytes were added to a confluent monolayer of 4×10^5 macrophages in flat bottomed dishes to arm the macrophages

[c] Mean of experiments performed in triplicate. The cytotoxicity of the armed macrophages was tested against SL2 cells

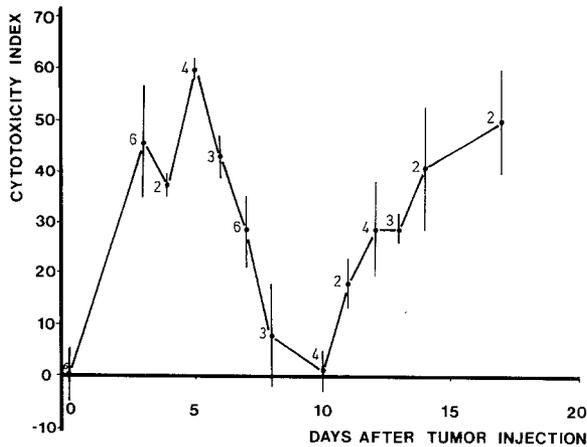[d] Cytotoxicity significantly different from the control macrophages ($P < 0.05$)

Fig. 4. Cytotoxicity of peritoneal macrophages during s.c. SL2 tumor growth. DBA/2 mice were injected with 10^4 SL2 cells/mouse (on day 0) in the right flank. On different days after immunization the peritoneal macrophages were harvested and the cytotoxicity was tested against SL2 cells. Mean ± SEM. The number of experiments, performed in triplicate, is shown in italics.
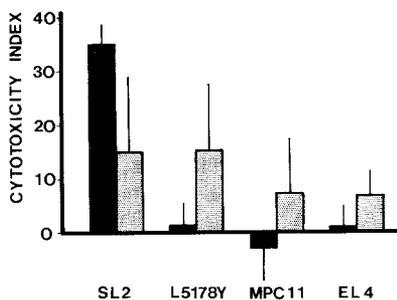

Fig. 5. Specificity of peritoneal macrophage cytotoxicity during SL2 growth in DBA/2 mice. DBA/2 mice were injected s.c. (on day 0) in the flank with 10^4 SL2 cells/mouse. On day 7 (■) and day 14 (▨) peritoneal macrophages were harvested and the cytotoxicity tested against various tumor cell lines: SL2 (DBA/2, H-2^d), L5178Y (DBA/2, H-2^d), MPC11 (BALB/c, H-2^d) and EL4 (C57BL, H-2^b). Mean ± SEM of 3 experiments performed in triplicate

SL2 cells, as the cytotoxicity against SL2 cells was not significantly different from the cytotoxicity against the other target cells.

Macrophage cytotoxicity induced by serum from tumor-bearing mice

As the tumor grew s.c. and the peritoneal macrophages became (specifically) cytotoxic i.p., we investigated whether the serum from tumor-bearing animals contained factors that could induce macrophage cytotoxicity. Serum was diluted with Fischer's medium and 0.6 ml was added to 8×10^5 macrophages (Costar, ∅ 16 mm). After 4 h the macrophages were washed and the cytotoxicity tested. As shown in Fig. 6, the serum was collected on different days after tumor injection and tested at a serum concentration of 75% or 25%. The presence of factors in the serum with the capacity to induce macrophages showed a similar biphasic pattern as the cytotoxicity of peritoneal macro-
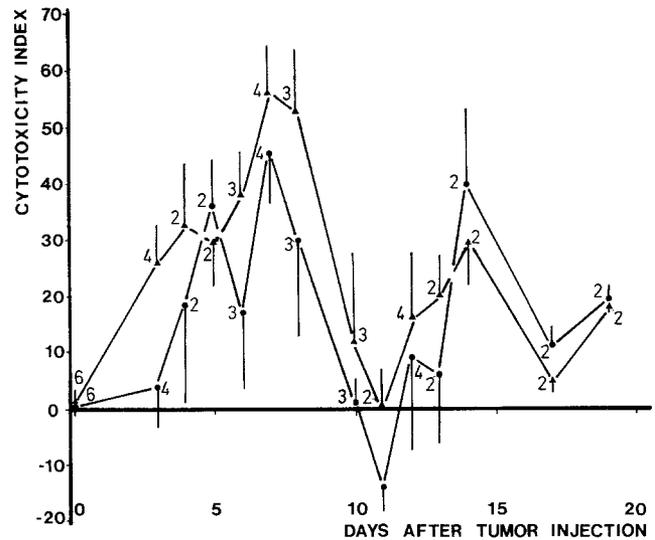

Fig. 6. The capacity of serum from SL2-bearing mice to induce cytotoxicity of normal peritoneal macrophages. DBA/2 mice were injected with 10^4 SL2 cells/mouse in the right flank on day 0. On different days after immunization serum was collected. This serum was added to a macrophage monolayer for 4 h. The serum concentration during incubation was 75% (▲) or 25% (●) in Fisher's medium. After incubation the monolayers were washed and the cytotoxicity tested against SL2 cells. Mean ± SEM. The number of experiments, performed in duplicate, is shown in italics
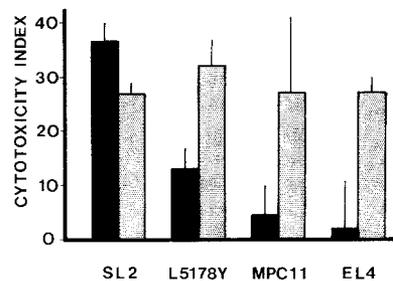

Fig. 7. Specificity of macrophage cytotoxicity induced by serum from SL2-bearing animals. DBA/2 mice were injected s.c. with 10^4 cells/mouse on day 0. On day 7 (■) and day 14 (▨) serum from tumor-bearing mice was collected and incubated at a concentration of 75% with normal peritoneal macrophages. After 4 h of incubation the monolayers were washed and the macrophage cytotoxicity tested against various tumor target cells. Mean ± SEM of 3 experiments performed in triplicate

phages (Fig. 4) during the growth of the SL2 tumor in DBA/2 mice. Normal mouse serum (day 0, Fig. 6) did not induce macrophage cytotoxicity (compared with normal macrophages not treated with serum).

To compare the cytotoxicity induced by serum with the cytotoxicity of the peritoneal macrophages from tumor-bearing mice, the specificity of the serum-induced macrophage cytotoxicity was tested. Serum obtained from mice bearing a SL2 tumor for 7 and 14 days was collected and the cytotoxicity induced by these sera tested against different tumor targets. As shown in Fig. 7, the cytotoxicity induced by serum from mice bearing a SL2 tumor for 7 days was specific for SL2 cells, whereas, the cytotoxicity induced by serum obtained 14 days after tumor grafting was nonspecific.
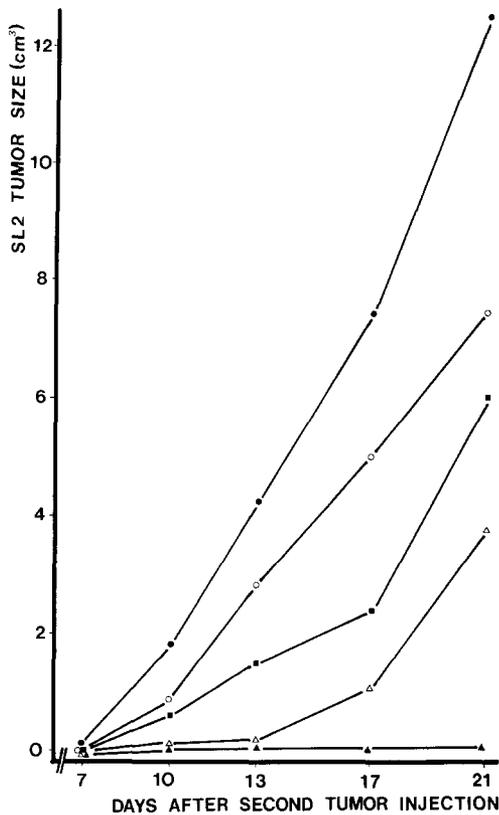
Fig. 8. Concomitant immunity in SL2-bearing DBA/2 mice against a second SL2 tumor graft. DBA/2 mice were injected with 10^4 SL2 cells in the right flank. On different days after this first tumor graft, a second SL2 tumor graft was given in the groin (10^4 cells/mouse). The growth of the second tumor graft was measured. The second tumor graft was given on the same day as the first SL2 graft (O) or 1 day (■), 4 days (△) or 7 days (▲) after the first graft. In the control only a tumor in the groin was given (●). Mean tumor size of 10 animals per group

Fig. 9. Concomitant immunity in SL2-bearing DBA/2 mice against a L5178Y tumor graft. DBA/2 mice were injected with 10^4 SL2 cells in the right flank. On the same day a L5178Y tumor (10^4 cells/mouse) was injected into the groin (O) or the L5178Y tumor was injected 1 day (■), 4 days (●) or 7 days (▲) after the SL2 tumor. In the control only a L5178Y tumor was given in the groin (△). The growth of the L5178Y tumor was measured. Mean tumor size of 10 animals per group

Concomitant immunity in SL2-bearing DBA/2 mice

To test whether SL2-bearing mice express an immune response against the SL2 tumor, concomitant immunity against a second SL2 tumor graft was tested. As shown in Fig. 8 the growth of a second SL2 tumor graft was retarded when injected simultaneously with the first tumor graft (day 0). When injected 1 or 4 days after the first tumor the second tumor graft showed even more pronounced growth inhibition. The second SL2 tumor did not develop at all when the second tumor graft was given 7 days (or after longer periods of time, data not shown) after the first graft.

To test the specificity of this concomitant immunity SL2 tumor-bearing animals were injected with a L5178Y (a syngeneic DBA/2) tumor graft. As shown in Fig. 9 the L5178Y tumor was hardly retarded in its growth when injected on the same day or 1 or 4 days after the (first) SL2 tumor. However, a second L5178Y tumor graft injected 7 days after the SL2 tumor did not grow at all. Also L5178Y cells injected more than 7 days after the SL2 tumor grafting did not develop into a tumor (data not shown). This indicated that in the first days (0–4 days) after SL2 grafting in DBA/2 mice the growth retardation of a second tumor graft was specifically directed against SL2 cells. From day 7 onwards a second tumor graft was, however, nonspecifically blocked in its growth.
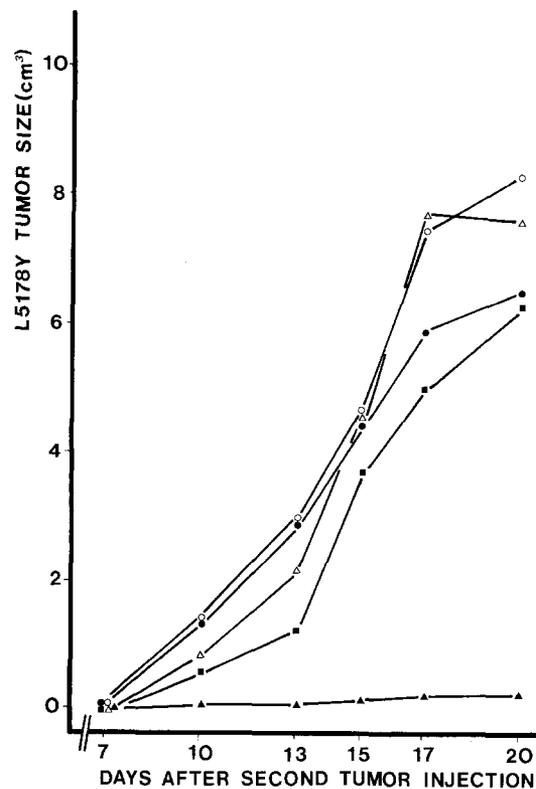
Lack of suppressive activity of serum from SL2-bearing mice

In many murine tumor systems serum contains "blocking factors". The presence of blocking factors may explain the low lymphocyte activity in lymphoid organs of SL2-bearing DBA/2 mice, whereas lymphocytes around the tumor can arm macrophages.

We therefore tested whether serum from tumor-bearing mice could block the macrophage arming activity of lymphocytes, from immunized DBA/2 mice. Neither preincubation of the sensitized lymphocytes, nor the presence of serum during the arming procedure reduced or enhanced the macrophage arming activity of the lymphocytes significantly (Table 5).

Lack of suppressive activity of lymphocytes from tumor-bearing animals

To test whether the lymphocytes from SL2-bearing DBA/2 mice were immune suppressive, their capacity to suppress the ability of SL2-immune lymphocytes to arm macrophages in vitro and the ability of SL2-immune lymphocytes to transfer tumor resistance against SL2 cells to naive animals was studied.

In vitro lymphocytes from tumor-bearing animals mixed with SL2-immune lymphocytes did not reduce the

Table 5. Test of suppressive activity in serum of SL2-bearing DBA/2 mice[a]

Days between tumor grafting and collection of serum	Cytotoxicity index[b]	
	SL2-immune lymphocytes preincubated in serum	Serum present during arming of the macrophages
Normal serum	44	51
4	51	49
7	57	52
11	53	46
13	61	44
16	41	50

[a] SL2-immune lymphocytes were used to render macrophages cytotoxic by direct arming (see Table 1). Before arming the immune lymphocytes were preincubated for 1 h at 37°C in 100% serum or the serum was added to the lymphocytes, macrophages, and SL2 cells during the arming process (end concentration of the serum: 20%). Normal serum obtained from nontumor-bearing DBA/2 mice was used as control

[b] Mean of a representative experiment performed in triplicate. The cytotoxicity of the armed macrophages was tested against SL2 cells

macrophage arming capacity of the SL2-immune lymphocytes. That is, spleen or lymph node lymphocytes from tumor-bearing animals did not inhibit the arming capacity of SL2-immune lymphocytes (data not shown).

As shown in Fig. 10A spleen lymphocytes obtained from DBA/2 mice immunized with SL2 cells significantly increased ($P<0.05$) the survival time of naive animals injected with SL2 cells. Lymphocytes from nonimmunized

mice were not able to do so. When SL2-immune lymphocytes were mixed with control lymphocytes or lymphocytes from tumor-bearing animals (bearing a s.c. SL2 tumor for 4, 7, 10, 14, 17, or 20 days) the survival time of the mice was not significantly different from mice injected with SL2 cells and SL2-immune lymphocytes only (Fig. 10B). In all groups of mice treated with SL2-immune lymphocytes about the same number survived (surviving more then 50 days, 20%–35%). This indicated that the lymphocytes of tumor-bearing mice could not suppress the antitumor activity of SL2-immune lymphocytes in vivo.

Transfer of tumor resistance with lymphocytes of tumor-bearing mice

To test whether spleen lymphocytes obtained from SL2 tumor-bearing mice could transfer tumor immunity to naive DBA/2 mice, DBA/2 mice were injected i.p. with 10^3 SL2 cells mixed with control spleen lymphocytes or spleen lymphocytes from tumor-bearing mice, collected 4, 7, 10, 14, 17, and 20 days after injection of the SL2 cells. Mice injected with SL2 tumor cells only died within 30 days. Injection of SL2 cells with control lymphocytes or lymphocytes from tumor-bearing animals did not increased the survival time, except for animals that received lymphocytes obtained from mice bearing a SL2 tumor for 14 days. In this latter group the mice showed a significantly increase survival time and 33% of the mice survived more than 50 days (Fig. 11 summarizes the data of 3 separate experiments). Spleen cells obtained from mice bearing a SL2 tumor for 14 days and purified over a nylon wool column [13] showed a similar capacity to transfer resistance as nonpurified spleen cells (data not shown). This indicat-
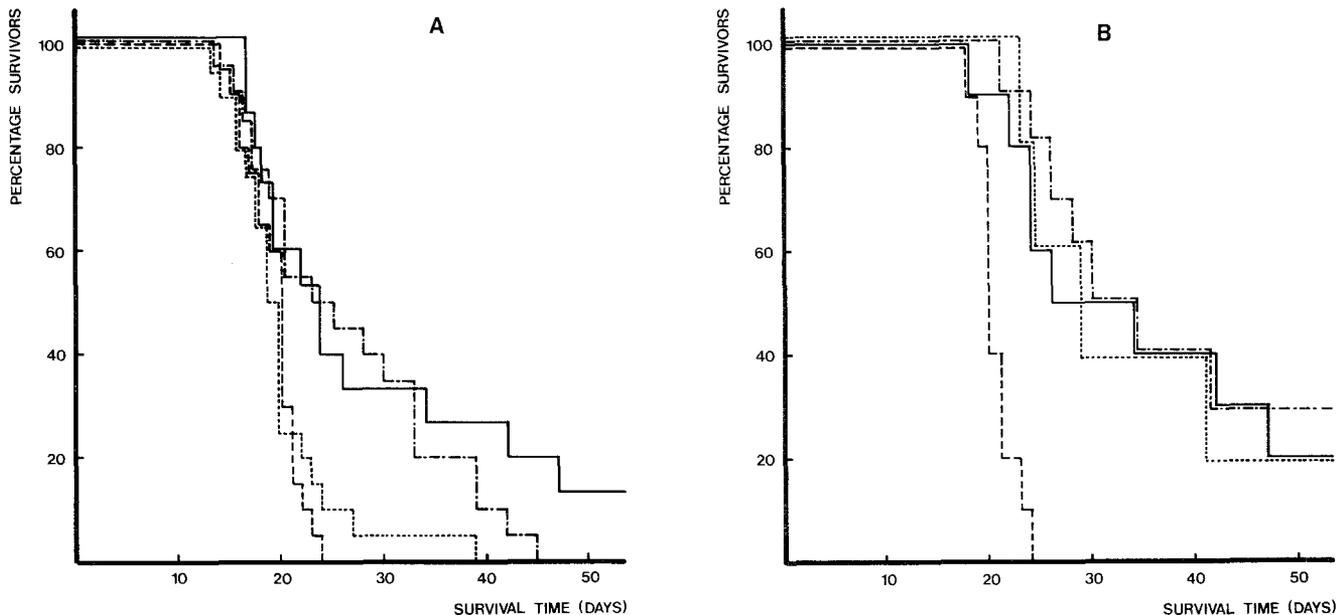

Fig. 10. The effect of spleen lymphocytes from SL2-bearing mice on the capacity of SL2-immune lymphocytes to transfer tumor resistance. **A:** Survival time of DBA/2 mice injected i.p. with 10^3 SL2 cells only (————), 10^3 SL2 cells and 10^6 control DBA/2 spleen lymphocytes (············), 10^3 SL2 cells and 10^6 SL2-immune spleen lymphocytes (from immunized DBA/2 mice; (—·—·—), or 10^3 SL2 cells, 10^6 SL2-immune spleen lymphocytes and 10^6 control spleen lymphocytes (————). Results of 4 separate experiments each with 5 animals per group. **B:** Survival time of DBA/2 mice injected i.p. with 10^3 SL2 cells only (————), 10^3 SL2 cells, 10^6 SL2-immune lymphocytes and 10^6 control lymphocytes (————), 10^3 SL2 cells, 10^6 SL2-immune lymphocytes and 10^6 spleen lymphocytes obtained from mice bearing a s.c. SL2 tumor for 7 days (—————) or for 20 days (············). Results were also obtained with spleen lymphocytes from mice bearing a s.c. SL2 tumor for 4, 10, 14, or 17 days. These results were similar to the results of lymphocytes from mice bearing the SL2 tumor for 7 or 20 days. Results of 2 separate experiments each with 5 animals per group
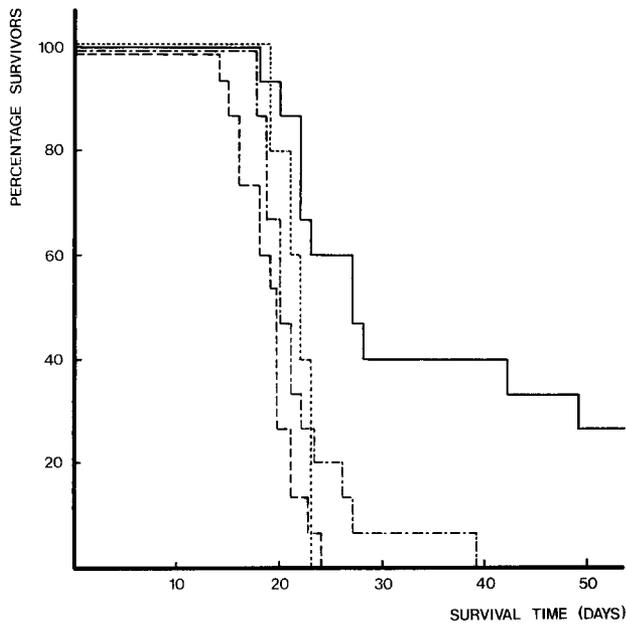
Fig. 11. Transfer of tumor resistance against SL2 cells by lymphocytes from SL2-bearing mice. Survival time of DBA/2 mice injected i.p. with 10³ SL2 cells and 10⁶ control spleen lymphocytes (———), 10³ SL2 cells together with 10⁶ spleen lymphocytes from mice bearing a SL2 tumor for 10 days (··········), 14 days (———) or 17 days (———). Spleen lymphocytes from mice bearing a tumor for 4, 7, or 20 days gave similar results as spleen lymphocytes from 10 or 17 day tumor-bearing mice. Results of 3 separate experiments each with 5 animals per group

ed that T-cells were responsible for the transfer of immune resistance by lymphocytes from mice bearing a tumor for 14 days.

Discussion

Tumor growth in vivo has been ascribed either to anergy of the immune system to the tumor cells or to immune suppression of the tumor reactive cells [17, 18, 25, 26]. Nonresponsiveness of the immune system due to the inability of the lymphocytes to recognize the tumor can be excluded in the DBA/2-SL2 system described in this paper, as the DBA/2 mouse can be immunized against the SL2 tumor [4, 29]. In addition, T-lymphocytes from the immunized DBA/2 mice can transfer resistance to tumor-bearing or naive animals [27, 29], indicating that these T-lymphocytes can mount an effective antitumor response. This antitumor response in the DBA/2 system has been shown to be dependent on cooperation between T-lymphocytes and macrophages [27, 28]. Finally, these SL2-immune T-lymphocytes can render macrophages specifically cytotoxic in vitro by the production of Specific Macrophage Arming Factor (SMAF, [7]. In vivo no cytotoxic peritoneal macrophages were found after i.p. immunization with irradiated SL2 cells. However, after a subsequent i.p. injection of nonirradiated SL2 cells, peritoneal macrophages were rapidly rendered cytotoxic. This macrophage cytotoxicity coincided with the clearance of SL2 cells from the peritoneal cavity [3]. The 4th day after injection of SL2 cells this, macrophage cytotoxicity appeared to be nonspecific, whereas on the 7th day the cytotoxicity became specifically directed to SL2 cells. As the peritoneal immune lympho-

cytes release SMAF that can render macrophages cytotoxic in vitro after contact with SL2 cells, it is likely that in a similar way peritoneal immune lymphocytes in vivo are triggered after i.p. injection of SL2 cells to arm macrophages specifically. In vivo these armed macrophages will remain in contact with the specific tumor cells until the injected SL2 cells are cleared. This contact between specifically armed macrophages and specific target cells will render the macrophage nonspecifically cytotoxic as has been described previously by Evans and Alexander [11]. When the tumor cells were cleared from the peritoneal cavity (days 3–4), macrophages became less cytotoxic, but the remaining cytotoxicity was more specific for SL2 cells. This suggests that the macrophages were still armed with SMAF but as there was no contact with the specific target cell the cytotoxic mechanism was "switched off". These data suggested a close correlation in immunized mice between tumor resistance and "specific" macrophage cytotoxicity induced by SMAF, that was produced by the immune lymphocytes after triggering with the SL2 cells. As we wanted to study why the antigenic SL2 tumor was not rejected in nonimmunized mice, we studied the lymphocyte reactivity against SL2 cells and the macrophage cytotoxicity in SL2-bearing DBA/2 mice.

Macrophage arming lymphocytes were found in tumor-bearing animals, around the subcutaneous tumor, but not in the lymphoid organs. This indicated that the lymphocytes were sensitized during tumor growth and remain localized at the site of the tumor. A similar homing of sensitized lymphocytes has recently been described in contact hypersensitivity [23, 24]. From the data in this paper it can be concluded that the lymphocytes at the site of the tumor release arming factors, as in the tumor-bearing mice peritoneal macrophages became cytotoxic. This cytotoxicity showed a biphasic pattern. The first phase (between days 3 and 7) of macrophage cytotoxicity was highly specific for SL2 cells, whereas the cytotoxicity in the second phase (after day 10) was not tumor specific. As the tumor grew subcutaneously, and the peritoneal macrophages were rendered cytotoxic we wondered whether we could detect factors in the serum that could render macrophages cytotoxic. These factors were indeed found. The time course of these factors showed a similar biphasic pattern as was found for the cytotoxicity of the peritoneal macrophages after tumor grafting. Besides, like the cytotoxicity of the peritoneal macrophages, the first phase of macrophage cytotoxicity induced by serum was specific and the second phase was nonspecific. Similar macrophage arming factors have been described by others during tumor growth [31, 32].

An injection of SL2 cells in SL2-bearing mice, changed the localization of the lymphocytes as macrophage arming lymphocytes were then also found in the spleen and the draining lymph node of the first SL2 graft. This experiment showed that the SL2-sensitized lymphocytes can leave the tumor site and circulate. Circulation of sensitized lymphocytes also explains the presence of concomitant immunity. As in other murine systems [14, 15, 16] the concomitant immunity in the SL2-bearing DBA/2 mouse would be divided into two phases. The first phase was tumor specific and has been ascribed to tumor specific T-cells in cooperation with macrophages [15, 16]. The second phase was nontumor specific and either due to a nonspecific activation of the macrophage system [15, 19] or to antimitotic agents released by the first tumor [12]. Both

phases seemed to occur in the DBA/2-SL2 system as well. The two phases of specific and nonspecific concomitant immunity closely paralleled the two phases of macrophage cytotoxicity. This suggests that the specific phase of concomitant immunity (between days 1 and 6) might be caused by sensitized lymphocytes and specifically armed macrophages, whereas, the nonspecific phase (after day 6) of concomitant immunity is caused by the nonspecific cytotoxicity of macrophages. This is in agreement with data from North et al. [19].

The similarity in concomitant immunity between this DBA/2-SL2 system and other nonlymphoid tumor murine systems (e.g., sarcomas, [14, 17] indicates that the immune reactions occurring in the DBA/2-SL2 system are not necessarily limited to lymphomas.

So, sensitization of lymphocytes to SL2 cells occurrs in SL2-bearing mice. Why then, is the tumor not rejected? We first studied whether the SL2-immune lymphocytes can be suppressed in their macrophage arming activity in vitro by factors in the serum from tumor-bearing mice or by lymphocytes from tumor-bearing mice. No inhibition or suppression by serum or lymphocytes from tumor-bearing mice on the macrophage arming activity of immune lymphocytes was found, suggesting that no suppressive mechanism for macrophage arming activity of the lymphocytes was present. This is in line with the finding that macrophage arming lymphocytes were present in the tumor-bearing mice as well.

The second possibility is that lymphocytes from tumor-bearing animals suppressed another activity required for tumor resistance. We therefore tested whether lymphocytes from tumor-bearing mice could suppress the capacity of immune lymphocytes to transfer tumor resistance to naive animals. However, spleen lymphocytes from tumor-bearing animals did not suppress the transfer of tumor resistance by SL2-immune spleen lymphocytes in a Winn-type assay. On the contrary, it was found that T-lymphocytes from DBA/2 mice bearing a SL2 tumor for 14 days, transfered resistance to naive animals as well. Lymphocytes obtained from DBA/2 mice injected 10 days or 17 days after injection of the SL2 tumor did not transfer tumor resistance. This was found in three separate experiments (Fig. 11). Why only spleen lymphocytes from day 14 transfered resistance is as yet unknown.

We have recently hypothesized that the immune response against tumor cells can be divided into an induction phase and an effector cell phase (Fig. 12, [6]). According to this hypothesis antigens on tumor cells can be recognized directly by T-cells, as was shown previously [8]. This T-cell-tumor cell interaction enables the T-cells to react with the release of a specific T-cell factor (e.g., SMAF). The factor can adhere to macrophages (or other cells). The specific factor can function as a bridge between the specific tumor cells and the macrophages (or other cells). The contact triggers the macrophage to kill the tumor cells. Subsequently, the macrophage will phagocytose the tumor cell debris and present tumor antigens. This will lead to the induction of helper T-cells and consequently to the further development of the immune response (the effector cell phase, Fig. 12). Other cells armed with the early specific T-cell factors might facilitate the development of the immune response as well. For instance, armed mast cells release serotonin after contact with the antigen. This facilitates the entry of lymphocytes and macrophages to the site of the
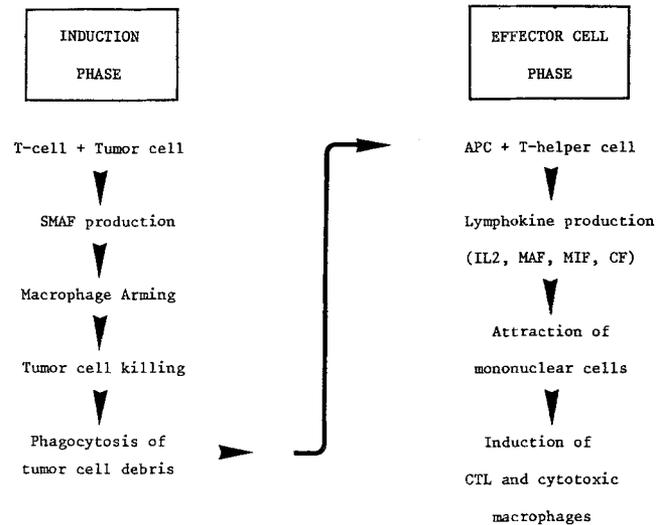

Fig. 12. Hypothesis for the immune response against tumor cells. The immune reaction is divided into two phases. An initiation phase in which the T-cells react directly with tumor cells, leading to the production of SMAF. Macrophages armed by SMAF can kill tumor cells. The tumor cell debris can be phagocytosed by antigen presenting cells (APC). These APC can induce the second phase of the response, the effector cell phase, by activating T-helper cells. These T-cells will produce lymphokines such as interleukin-2 (IL-2), Macrophage Activating Factor (MAF), Migration Inhibitory Factor (MIF) and Chemotactic Factor (CF). These lymphokines will attract mononuclear cells to the site of the tumor and activate cytotoxic T-cells (CTL) and macrophages

tumor [1]. This latter suggestion of a role for a specific T-cell factor and mast cells in the initiation of an immune response, was recently put forward by Askenase and Van Loveren [1].

It is of interest to compare the data described in this paper on SL2-bearing DBA/2 mice with the hypothesis depicted in Fig. 12. Apparently the induction phase is not affected in the tumor-bearing animal, as macrophage arming lymphocytes are present, SMAF is present in the serum, and specifically cytotoxic macrophages are present in the peritoneal cavity. This suggests that the induction phase develops in contrast to the effector cell phase. This may be due to two reasons. In the first place it is possible that agents produced by the tumor cells or activated macrophages inhibit the induction of helper T-cells by the antigen presenting cells, which will prevent the development of the effector cell phase. In the second place it is possible that macrophages in syngeneic tumor systems cannot present an antigenic moiety from killed tumor cells. In this latter case resistance to the tumor only occurs when the production of specific T-cell factors and the subsequent arming of macrophages (initiation phase) is strong enough to reject a challenge of tumor cells directly. This situation might occur after hyperimmunization with irradiated tumor cells and subsequent challenge with nonirradiated cells. In the DBA/2-SL2 system it has been shown that two subsequent immunizations with irradiated cells are required to induce immunity to SL2 cells, as after one injection with irradiated SL2 cells DBA/2 mice were unable to reject a challenge with nonirradiated SL2 cells [7]. When the mice, immunized twice i.p. with 10^7 irradiated cells, were challenged i.p. with nonirradiated cells (5×10^6 cells/

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mouse) a rapid production of SMAF, as detected by the occurrence of specifically cytotoxic macrophages, takes place. The tumor cells were killed within 2–3 days. These killed tumor cells are phagocytosed and the macrophages should then be able to present antigens. However, triggering of T-helper cells by antigen presenting cells should result in the production of lymphokines, that will attract mononuclear cells. This expected influx of mononuclear cells does not occur in immunized DBA/2 mice [3], suggesting that lymphokine production is small or absent. Presently, we are studying whether DBA/2 macrophages indeeed lack the capacity to present SL2 antigens to DBA/2 lymphocytes.

In conclusion, the data from the DBA/2-SL2 system suggest that during SL2 tumor growth the immune system is reacting to the SL2 cells but does not mount a proper rejection reaction. The lack of development of proper effector cells seems to be due to an inability of the immune system to react to the antigenic tumor with sufficient numbers of lymphocytes rather than to immune suppression of the tumor resistance.

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