Europ. J. Cancer Vol. 16, pp. 1417–1426

0014–2964/80/1101–1417 $02.00/0

Changes in Spleen Morphology and Lymphoid Cell Activity During Tumor Progression*

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Abstract—*Morphologic alterations in the spleen and dynamic changes in the biologic activity of its cellular components occur concomitant with the neoplastic progression of a murine methylcholanthrene induced fibrosarcoma. Spleen size, weight and cell number increased with tumor growth. Using the local adoptive transfer assay (LATA), spleens from tumor bearing mice in the early and late stages of neoplastic growth were shown to possess non-specific, tumor-facilitating cells which were radioresistant, phagocytic and adherent, presumably macrophages. On the other hand, spleens from mice in the intermediate and late stages of tumorigenesis displayed specific, tumor-neutralizing cells, which were radiosensitive and Thy 1.2 positive, presumably T-cells. Thus, a balance of tumor-facilitating and neutralizing cells may determine neoplastic progression in susceptible, syngeneic hosts.*

INTRODUCTION

ALTHOUGH cytotoxic lymphocytes [1, 2], macrophages [3, 4] and other killer cells [5] have been demonstrated in tumor-bearing hosts, most animals succumb to unabated neoplastic progression. Three immune mechanisms antagonize the cytotoxic effects and promote tumor escape: suppressor T-lymphocytes [6–12], B-lymphocytes [13–15] and macrophages [16–21].

Sinusoidal phases of tumor-facilitating and tumor-neutralizing spleen cell activities were previously reported in local adoptive transfer assays (LATA) 2, 6, 9, 12, and 15 days after mice were treated with crude 3 M KCl extracts. These antagonistic activities were attributed to the effects of distinct tumor-facilitating and tumor-neutralizing antigenic fractions [22, 23]. Isoelectric focusing of crude 3 M KCl extracts yielded fraction 1, which induced specific tumor facilitation, and fraction 15, which evoked specific tumor resistance. The two opposing antigens were shown to individually stimulate corresponding facilitating or neutralizing activities of splenic lymphoid cells [24] in the LATA. The present study was undertaken to examine cellular events in the spleens of tumor-bearing animals, in order to ascertain whether a similar sinusoidal progression was present in mice sensitized by viable neoplastic cells as opposed to 3 M KCl extracts. The studies revealed not only morphological alterations, but also dynamic changes in spleen cell activit during tumor growth which were akin to those observed after soluble antigen treatment.

Accepted 17 March 1980.
*Supported by Grant IM-62E from The American Cancer Society.

MATERIALS AND METHODS

Animals and tumors

Tumors were induced in female C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) by subcutaneous (s.c) inoculation of 175 μg of 3-methylcholanthrene (MCA), as described previously [24]. Two antigenically distinct, non-cross reactive tumors, MCA-F and MCA-D, were maintained by serial SC passage in syngeneic hosts.

Tumor cell populations

Tumor nodules were excised and single cell suspensions prepared by treatment of minced tissue with 0.25% trypsin–Hanks solution. The resultant tumor cell suspension was washed three times by centrifuging at 200 ***g*** for 10 min, and then resuspended in minimum

essential medium (MEM). Tumor cell viability as assessed by trypan blue exclusion was generally over 90%.

Preparation of spleen cells

Spleens pooled in MEM containing penicillin and streptomycin were finely minced before being pressed between two sterile glass slides and passed through a 160-mesh stainless steel screen. Resultant suspensions were washed twice with plain MEM at 200 ***g*** for 10 min. Viability was greater than 90% by trypan blue exclusion.

Spleen changes

Spleen length and weight (3 spleens/group) were measured with calipers and on a Mettler PT 320 balance. Spleens from normal and tumor-bearing mice were fixed in 10% formalin solution. Histologic sections were examined after staining with hematoxylin and eosin (H–E). Spleens of each group were pooled and minced. The total white and red blood cell counts of the spleen cell suspension were determined using a hemocytometer; differential counts were performed on smears which had been fixed with ethanol and stained with the Giemsa reagent. The ratio of WBC to RBC was calculated by the formula: total spleen cell number × % (WBC or RBC).

Fractionation by phagocytosis

To 1×10^8 spleen cells in 10 ml of MEM containing 10% fetal calf serum was added ten volumes of carbonyl iron suspension (GAF Corp., New York, N.Y.). After incubation for 1 hr at 37°C on a rocking platform, the cell suspension was poured into a Petri dish sitting on a strong magnet to sediment cells which had phagocytized the iron powder. The supernate was removed with a Pasteur pipette. This procedure was repeated 4–5 times to obtain a cell population incapable of phagocytosis.

Fractionation by adherence to plastic dishes

Following the treatment with carbonyl iron/magnet, the spleen cell suspension (2×10^6/ml) was incubated in a plastic Petri dish at 37°C for 1 hr. Non-adherent cells were collected after gentle rocking. The procedure was repeated a second time and the resultant non-adherent cell suspension washed five times with plain MEM and sedimented at 200 ***g*** for 10 min.

T-Cell depletion

Depletion of Thy-1-bearing spleen cells was achieved by treatment with anti-Thy 1.2 antiserum and complement. Spleen cells (5×10^7 cells ml RPMI) from tumor-bearing or age-matched normal mice were incubated for 30 min at 4°C with 1 ml of a 1:500 dilution of monoclonal IgM anti-Thy 1.2 antibody (kindly supplied by Dr. Hammerling, Sloan–Kettering Institute, New York). After the addition of 1 ml of a 1:15 dilution of lyophilized rabbit serum (Low-Tox-M Rabbit Complement, Cedarlane Laboratories) incubation was continued for an additional 30 min at 37°C in a shaking bath. The proportion of viable lymphocytes was determined by trypan blue exclusion. Cells treated with only complement served as the control. The efficacy of T cell removal anti-Thy 1.2 antiserum treatment was confirmed by a greater than 90% reduction in lymphocyte proliferation following stimulation with PHA.

Irradiation

Spleen cells from tumor-bearing or normal mice were subjected to a single 700 rad dose from a ^{137}Cs gamma source at an absorbed dose rate of 5800 rad/min.

Local adoptive transfer assay (LATA)

Spleen cells from mice inoculated with 1×10^5 MCA-F cells 3, 6, 9, 15 and 30 days prior to transfer were assessed for activity by LATA. The LATA mixtures consisted of 10^7 spleen cells, which were pooled from 3–5 donors of each group, and 10^4 MCA-F cells propagated *in vivo*. Following incubation at room temperature for 1 hr, the mixtures were inoculated s.c. into groups of 10 syngeneic normal recipients. Controls received tumor cells admixed with normal spleen cells. The specificity of the spleen cell activity was determined by comparing the outgrowth of mixtures of MCA-F tumor-bearer spleen cells in the presence of either MCA-F, of the antigenically distinct MCA-D, cells. Neoplastic growth was monitored by serial measurement of two tumor diameters with vernier calipers. The data were expressed as the mean tumor diameter. The significance of the percentage change in tumor growth was determined by chi-square comparison of growth values when cells were admixed with normal vs tumor-bearing spleen cells, and by Student's *t*-test analysis of individual tumor diameter measurements.

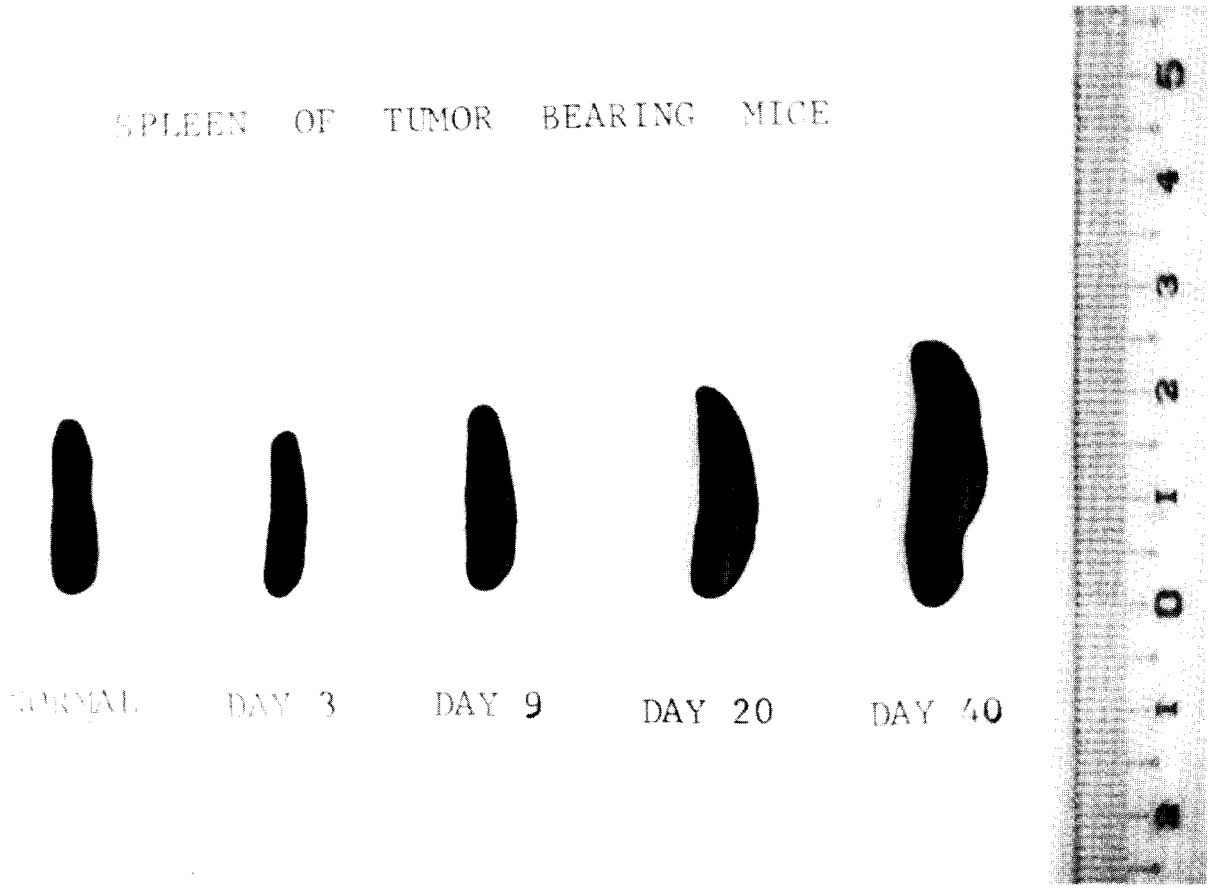

Fig. 1. Changes in spleen size attendant to MCA-F progression. Spleens were excised on the indicated day from hosts previously inoculated with 10^5 *viable MCA-F cells.*

RESULTS

Morphologic changes of spleen during tumor growth

Splenic length (Figs. 1 and 2), weight (Fig. 3A), total cellular content (Fig. 3B) and number of white blood cells (Fig. 3C) increased concomitantly with the progression of MCA-F. Although the number of red blood cells (Fig. 3D) decreased initially during tumor growth to 15 mm diameter, it increased thereafter. Histologic examination revealed that the increase in spleen size was due to white pulp hyperplasia. There was no evidence of tumor cell invasion in the spleen. Furthermore, MCA-F invasion at 30 days after inoculation was excluded as the cause of splenomegaly by the failure of inoculation of 4×10^8 cells from spleens of tumor-bearing hosts to display neoplastic outgrowth in syngeneic normal mice.

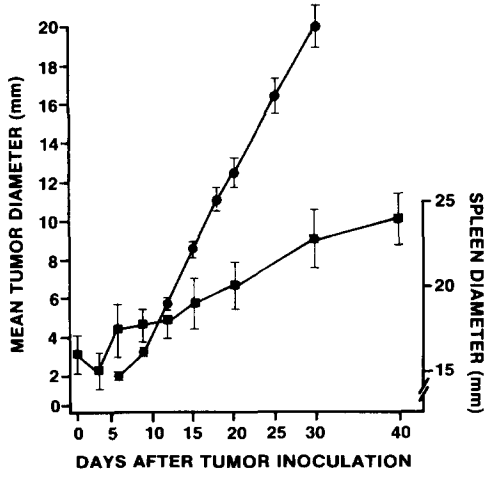

Fig. 2. Concurrent increase in tumor and spleen size in mice inoculated with 10^5 MCA-F cells. Serial measurements of neoplastic diameter on a group of 30 animals were performed at 3-day intervals. Following each tumor measurement, three mice were sacrificed and the length of their spleens determined.

Characterization of tumor-facilitating cells in early stages of tumorigenesis

The immunological effects of spleen cells from mice bearing MCA-F tumors (TBsp) for three days were studied by LATA. In the early stage of tumorigenesis, TBsp from animals bearing MCA-F significantly facilitated the growth of both MCA-F and MCA-D tumors compared to spleen cells from normal donors (Table 1). Thus, tumor-facilitating cells generated in the early stage of tumor growth were non-specific. The facilitating activity was resistant to irradiation with 700 rad. These tumor-facilitating spleen cells were fractionated using the carbonyl-iron/magnet procedure followed by adherence to plastic Petri dishes prior to LATA. Figure 4 shows that depletion of phagocytic, adherent cells abrogated the tumor-facilitating activity. The non-specific, tumor-facilitating activity appears to be due to a radioresistant, phagocytic, adherent cell population, presumably macrophages.

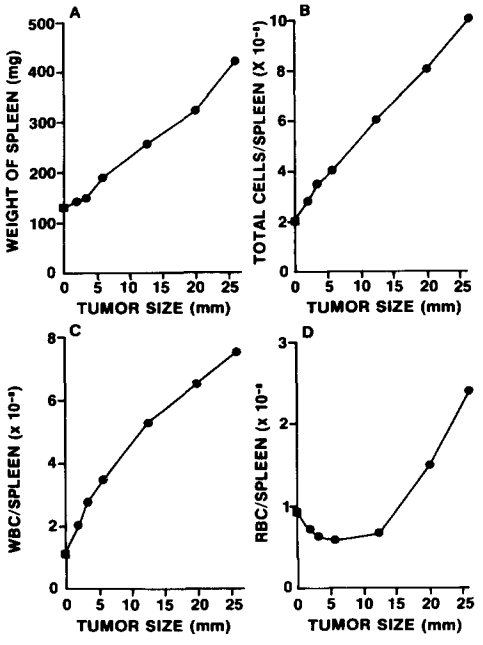

Fig. 3. Cellular changes in the spleens of mice with progressive MCA-F neoplasms. At the indicated intervals after inoculation of 10^5 MCA-F cells, three spleens were harvested, (A) weighed, (B) total cells estimated, and differential of white (C) and red (D) blood cell numbers determined from smears stained with Giemsa reagent.

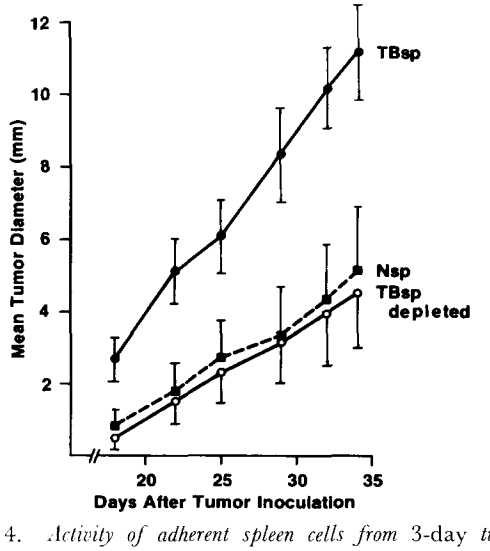

Fig. 4. Activity of adherent spleen cells from 3-day tumor bearing hosts. Tumor bearer spleen cells were incubated in Petri dishes for 1 hr, and the non-adherent fraction replated and incubated for an additional hour. Non-adherent 'depleted' tumor-bearer spleen cells (○—○), unfractionated normal (■----■) and unfractionated tumor bearer (●—●) spleen cells were admixed with 10^4 MCA-F cells (1000:1, effect:target) and injected s.c. into ten syngeneic hosts. Serial measurements of tumor diameter were used to estimate effect of the spleen cell populations upon neoplastic outgrowth.

Table. 1. Effect of irradiation and target cell antigens upon the tumor facilitating activity of spleen cells from 3-day *tumor bearers*

Spleen cell donor	LATA tumor target	Tumor diameter (mm ± S.E.M.) at 26 days after LATA*
None	MCA-F	8.0 ± 1.2
Normal	MCA-F	6.5 ± 1.2
TBsp	MCA-F	13.1 ± 1.0† ($P<0.001$)
TBsp-x†	MCA-F	13.8 ± 1.1§ (NS)
None	MCA-D	8.4 ± 1.4
Normal	MCA-D	6.4 ± 0.6
TBsp	MCA-D	14.5 ± 0.5‡ ($P<0.001$)
TBsp-x	MCA-D	15.8 ± 1.0§ (NS)

*Three days after inoculation of 10^5 MCA-F cells into each of 20 C3H/HeJ mice, spleen cells were harvested. Normal and tumor bearer spleen cells were individually mixed with 10^4 MCA-F or with 10^4 MCA-D cells and inoculated s.c. into groups of ten syngeneic hosts. The average of the mean tumour diameters ± S.E.M. the stipulated day after transfer are shown.

†Significance of Student *t* comparison with recipients of normal spleen cells.

‡Spleen cells were exposed to 700 rad of gamma irradiation prior to use in the LATA.

§Significance of difference between untreated tumor bearer and X-irradiated tumor bearer spleen cells.

Table 2. Effect of irradiation upon the tumor neutralizing activity of spleen cells from 9-day *tumor bearers*

Spleen cell donor	LATA cell target	Tumor diameter (mm ± S.E.M.) at 22 days after LATA*
None	MCA-F	10.2 ± 1.0
Nsp	MCA-F	9.5 ± 1.2
TBsp	MCA-F	4.7 ± 1.0† ($P<0.01$)
TBsp-x	MCA-F	8.1 ± 1.2‡ ($P<0.05$)
None	MCA-D	6.0 ± 0.8
Nsp	MCA-D	5.3 ± 0.7
TBsp	MCA-D	5.8 ± 1.1 (NS)
TBsp-x	MCA-D	6.7 ± 1.2 (NS)

*See legend for Table 1.

†Comparison with normal spleen cells.

‡Comparison with non-irradiated tumor bearer spleen cells.

Table 3. Effect of anti-θ treatment of TBsp from 9-day *tumor bearers upon tumor neutralizing activity*

Spleen cell donor	Treatment*	Mean tumor diameter (mm) ± S.E.M. at 27 days of LATA	*P*
None	None	12.8 ± 1.2	
Nsp	None	12.9 ± 1.0	
	C′	10.0 ± 1.2	NS
	Anti-θ ± C′	11.9 ± 1.4	NS
TBsp	None	3.3 ± 1.5	<0.001
	C′	3.2 ± 1.0	<0.001
	Anti-θ ± C′	9.2 ± 1.8	NS

*Spleen cells derived either from normal animals or from hosts 9 days after tumor inoculation were treated *in vitro* with monoclonal anti Thy 1.2 antiserum and rabbit complement prior to LATA in syngeneic normal hosts as described in legend for Table 1.

Characterization of tumor-neutralizing cells in intermediate stages of tumorigenesis

In the intermediate stage of tumor progression, namely nine days after MCA-F inoculation, there was a TBsp population which specifically neutralized neoplastic outgrowth. This activity was radiosensitive (Table 2). Since it was eliminated by the anti-Thy 1.2 treatment, neutralizing activity of TBsp is probably mediated by a tumor-specific, radiosensitive T-cell subpopulation (Table 3).

Characterization of tumor-neutralizing and facilitating cells in late stages of tumorigenesis

In the late stages of tumor growth, namely 30 days after MCA-F inoculation, TBsp not only neutralized MCA-F growth, but also facilitated MCA-D growth in LATA (Table 4). Irradiation of TB spleen cells abrogated the specific tumor-neutralizing effect, but did not alter the non-specific, tumor facilitating activity. Thus, two components are generated in the late stages of tumor growth: (1) specific, radiosensitive tumor-neutralizing cells and (2) non-specific, radioresistant tumor-facilitating cells.

To characterize these populations, spleen cells fractionated using the carbonyl-iron/magnet procedure followed by adherence to plastic Petri dishes were assessed by LATA. When a spleen cell population which neutralized MCA-F ($P<0.001$) but facilitated MCA-D ($P<0.01$) was depleted of adherent, phagocytic elements, there was a significant increase in neutralizing activity against the MCA-F tumor ($P<0.005$) with abrogation of the facilitating activity against MCA-D ($P<0.005$) (Table 5). On the other hand, depletion of adherent, phagocytic cells from the normal spleen cell population did not alter the outgrowth of MCA-F or MCA-

Table 4. Specificity and radiosensitivity of spleen cells from mice bearing MCA-F tumor for 30 days

LATA target cell	Spleen cell donor	Mean tumor diameter (mm) ± S.E.M. at 30 days after LATA
MCA-F	None	12.2 ± 1.4
	Nsp	11.6 ± 1.3
	Nsp-x	10.4 ± 1.2 (NS)
	TBsp	6.8 ± 0.8† (<0.002)
	TBsp-x	9.7 ± 0.9‡ ($P<0.05$)
MCA-D	None	8.7 ± 1.0
	Nsp	8.7 ± 0.8
	Nsp-x	9.0 ± 0.9 (NS)
	TBsp	12.0 ± 0.7† ($P<0.005$)
	TBsp-x	11.5 ± 0.3‡ (NS)

*See Legends for Table 1.
†Significance of difference between Nsp and TBsp.
†Significance of difference between TBsp and TBsp-x.

Table 5. Effect of depletion of adherent and phagocytic elements from spleen cell populations at 30 days *after* MCA-F *inoculation*

LATA target cell	Spleen cell	Mean tumor diameter (mm ± S.E.M.) at 27 days after LATA*	
MCA-F	None	10.0 ± 1.1	NS
	NSP	10.1 ± 1.1	—
	Nsp (depleted)	10.1 ± 1.2	NS
	TBsp	4.8 ± 0.9	<0.001
	TBsp (depleted)	1.9 ± 0.0 ($P<0.005$)‡	<0.001
MCA-D	None	8.7 ± 1.0	NS
	Nsp	8.7 ± 0.8	—
	Nsp (depleted)	9.0 ± 0.9	NS
	TBsp	12.0 ± 0.7	<0.01
	TBsp (depleted)	7.7 ± 0.6 ($P<0.005$)‡	NS

*See Legends for Table 1.
†Significance by Student *t* comparison with Nsp.
‡Significance of difference between TBsp and TBsp (depleted).

D. Thus, facilitating cells present in the TBsp population suppress the activity of tumor-neutralizing cells presumably by dampening T-cell mediated immunity.

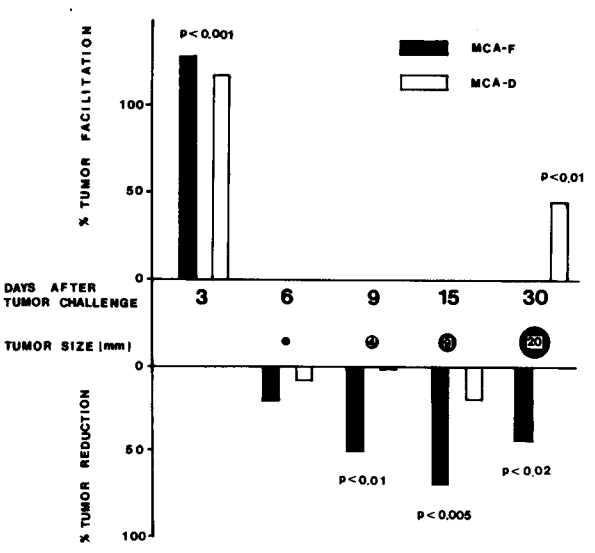

Fig. 5. Evolution of the immune response of spleen cells obtained at various times after tumor inoculation. Unfractionated tumor bearer and normal spleen cell populations were assessed for their effect upon tumor growth in the LATA. Spleen cells from MCA-F inoculated hosts were admixed with 10^4 *MCA-F cells and with* 10^4 *MCA-D cells* (1000:1) *and the mixtures injected s.c. into ten syngeneic recipients. Percentage reduction or facilitation of tumor growth was calculated as described previously* [22].

Dynamic changes of spleen cell activity

Figure 5 summarizes the dynamic changes in spleen cell activity measured by serial LATA during tumor progression. Spleen cells from mice 3, 6, 9, 12, 15 and 30 days after tumor inoculation were separately admixed with neoplastic cells bearing either specific (MCA-F) or non-crossreactive (MCA-D) antigens. The percentage facilitation or reduction of tumor outgrowth was derived from data presented in Tables 1–5. Spleen cells from early stage tumor-bearers at three days facilitated the growth of both MCA-F and MCA-D tumors. Spleen cells during the intermediate stage of tumor growth at 9–15 days retarded MCA-F, but not MCA-D progression. At 30 days, the spleen cells retained their capacity to neutralize MCA-F, but facilitated the growth of the antigenically distinct MCA-tumor. The results demonstrate sinusoidal changes in the facilitating and neutralizing activities of the tumor-bearer spleen cell population.

DISCUSSION

Splenomegaly accompanies tumor progression and appears to result from white pulp hyperplasia with increased numbers of lym-

phocytes and large mononuclear cells. Neither histological examination nor transplantation of spleen cells into syngeneic hosts revealed neoplastic infiltration. Yabello *et al.* [25] noted splenomegaly during MCA-tumor growth in C3H/HeJ mice. Padarathsingh *et al.* [26] found increased numbers of splenic macrophages, as detected by esterase staining, to correlate with tumor-size in mice bearing the ADJ-PC5 plasmacytoma. The increase in splenic cellularity may be due either to local lymphoreticular proliferation or to a recruitment of cells from peripheral lymphoid organs.

The data presented herein revealed that the spleens of mice bearing MCA-F neoplasms contain cells mediating both tumor-facilitatation and tumor-neutralization. Non-specific tumor-facilitating cells, probably macrophages, were present during the early and late stages of tumorigenesis. Specific tumor-neutralizing cells, probably T-cells as demonstrated by sensitivity to Thy 1.2 serum, were detected during the intermediate and late stages of tumor growth. Thus, the relative activities of tumor facilitating cells and neutralizing cells may determine host resistance to neoplastic progression.

These results were consistent with our previous study demonstrating that electrophoretically distinct antigens in crude soluble 3M KCl extracts stimulate lymphoid populations with tumor-facilitating and immunoprotective activities [23]. Comparison of the kinetics of induction of these populations after the administration of antigenic extracts with that after neoplastic cell inoculation revealed two differences, the first of which was evident in the onset and durability of the responses. Animals exposed to viable tumor cells displayed facilitation at 3 days and resistance at 6 days; animals immunized with extracts displayed facilitation at 6 days and resistance at 9 days. The resistance phase persisted at 30 days in the former setting but was lost by 15 days in the latter case. Thus the extracts showed a delayed onset and abbreviated duration of both the resistance and facilitation phases. Secondly, at least a portion of the facilitating activity induced by the extract fraction I was mediated by lymphoid cells, whereas macrophages appeared to facilitate neoplastic growth in tumor-bearers.

Using a microcytotoxicity assay *in vitro* Lamon *et al.* [27] observed a cyclical course of immunological events. Specific cytotoxic murine lymphocytes appeared during the early stage of tumor-bearing, but were lost as the tumor grew. No facilitating activity was detected. Recently, Huber *et al.* [28] reported that spleen cells from Fisher rats immunized i.p. with syngeneic mammary adenocarcinoma cells displayed a biphasic pattern of T-cell-mediated cytolytic and suppressor responses. While cytolytic cells generated in early stages were nonspecific, those observed in later stages were antigen-specific. Effector and suppressor lymphoid cells were recently demonstrated by Berczi and Sehon [29] in tumor-bearing guinea-pigs 2 weeks after s.c. implantation of MCA-induced sarcomas. Yu *et al.* [30] reported suppressor and neutralizing spleen cell populations in tumor-bearing mice, suggesting that the appearance of suppressor cells, and the appearance and disappearance of neutralizing cells, correlated with the number of inoculated tumor cells and the duration of neoplastic progression. Specific suppressor T-cells have been detected in the intermediate stages of tumor growth by Fujimoto and colleagues [6, 7]. The present experiments suggested that macrophages serve to suppress host resistance and thereby facilitate neoplastic outgrowth since the active population was radioresistant, phagocytic, and adherent. However, the experiments do not exclude the concomitant participation of a T cell subpopulation. The apparent supremacy of macrophage mediated facilitation in the experiments reported herein compared to suppressor T cell activity in the work of Fujimoto [6, 7] may relate to differences in tumor antigenicity, immunization method, choice of lymphoid organ and detection technique.

In the MCA-F model, macrophages appear consistently to antagonize host resistance to neoplastic outgrowth. For example, depletion of adherent, phagocytic elements significantly potentiated the neutralizing activity of spleen cells from late stage tumor-bearers, which already displayed predominant tumor-neutralization. The tumor-facilitating macrophages documented herein may represent an *in vivo* correlate of the *in vitro* suppressive activity previously reported by others. Poupon [21] noted a non-specific, adherent, non-T cell splenic population from C3H mice bearing MCA-induced fibrosarcomas to suppress *in vitro* lymphoid cell proliferation following mitogen stimulation. Pope [20] reported that spleen cells from DBA/2J mice bearing an MCA-induced rhabdomyosarcoma suppressed the *in vitro* mitogenic responses of lymphoid cells. These cells were depleted by passage through nylon wool, by treatment with carbonyl iron, and by adherence to plastic, but not by

exposure to anti-Thy 1.2. Oehler [19] described a population of adherent, phagocytic spleen cells, which suppressed the mixed lymphocyte culture reaction, to be resistant to treatment with ATS and complement, as well as radiation. Eggers [17] reported that tumor-bearing mice contained non-specific suppressor cells, presumed to be macrophages, which inhibited *in vitro* immunization against alloantigens. Finally, Kirchner *et al.* [31] suggested that suppressor cells capable of inhibiting *in vitro* DNA synthesis by T-lymphocytes were of the monocyte/macrophage series. The present experiments represent the initial report that suppressor, tumor-facilitating macrophages alter neoplastic progression *in vivo*.

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