

EVIDENCE OF SUPPRESSOR CELL ACTIVITY IN SPLEENS
OF MICE BEARING PRIMARY TUMORS INDUCED BY
MOLONEY SARCOMA VIRUS*

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According to the immune surveillance theory, tumors may arise because of a defect in the immune system (1, 2). Indeed, there are situations in which immunosuppression leads to an increased incidence of neoplasia. However, in most cases in which defects of the immune system have been noted in patients and animals with spontaneous tumors, it is impossible to decide whether these are secondary in nature rather than preceding tumor development. It therefore seems of considerable interest to perform functional analyses of lymphocytes in well characterized experimental tumor systems in order to better define alterations of the immune system, associated with the development of tumors. These studies may detect two types of defects: those preceding tumor development, and those accompanying their growth. The latter, although not providing support for the major tenet of the immune surveillance theory, may have considerable relevance to the crucial problem of tumor immunology as to why a growing tumor cannot be rejected despite the recognition of tumor-associated antigens (see reviews of these problems in references 3-6).

We have initiated a series of experiments where a variety of lymphocyte functions are analysed during the ontogeny of murine tumors (references 7 and 8, and footnote 1). Functional tests of immunity to tumor-associated antigens were applied, as well as tests of general immunocompetence. The latter seem to be able to unravel additional alterations of the immune system of tumor-bearing hosts, which cannot be detected with ease by the specific assays of tumor immunity. The *in vitro* stimulation by mitogens, such as phytohemagglutinin (PHA),² has proven useful for evaluating reactivity of

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² Abbreviations used in this paper: B6, C57BL/6N mice; BUDR, 5-bromo-2'-deoxyuridine; C', guinea pig complement; LPS, bacterial lipopolysaccharide (endotoxin); LN, lymph node(s); MSV, Moloney sarcoma virus; PFC, plaque-forming cells; PHA, phytohemagglutinin; SRBC, sheep red blood cells; TdRH3, [³H]thymidine.

lymphocytes in patients with malignancies and animals with experimental tumors (9–13). We and others have recently observed a markedly depressed PHA response in spleens of mice carrying Moloney sarcoma virus (MSV)-induced tumors (8, 14), and have suggested that this is not an intrinsic defect of the PHA responsive T cells, but rather may be due to a suppressive effect by another cell. In the present investigation we have combined a number of experimental approaches which indeed demonstrate that activation of such suppressor cells occurs in MSV spleens.³

Materials and Methods

Animals and Tumor Systems.—C57BL/6N (B6) and Balb/C mice were obtained from the Division of Research Services, NIH, Bethesda, Md. Primary MSV tumors were induced by intramuscular injection of 0.05 ml of MSV into the right hind leg of 10- to 14-wk old B6 mice, as previously described in detail (7). In this system tumors reach a maximum size between 12 and 16 days after virus inoculation and then usually regress after 21 days. All experiments of this study were performed between 12 and 16 days. In selected experiments two additional tumor systems were used. In one of these, tumors were induced by intramuscular injection of a variant of the MSV (Gz-MSV(M)) which causes progressively growing tumors in adult B6 mice. Dr. A. Gazdar, NIH, kindly provided this virus, the biological characteristics of which will be described elsewhere. Spleens of these mice were serially tested at intervals of 5 days during the entire course of tumor development. In addition, the RBL-5 lymphoma of B6 mice (15) was utilized, a Rauscher leukemia virus-induced ascitic tumor. It had been maintained in serial passage for many years and killed B6 mice within 15–20 days. Spleens of these were investigated 7–10 days after intraperitoneal tumor cell injection. Nude (nu/nu) mice which had gone through 2–3 backcross generations with Balb/C mice, were obtained through the courtesy of Dr. Carl Hansen, Division of Research Services, NIH.

In Vitro Lymphocyte Culture Technique.—Spleens, and in some instances lymph nodes (LN), were aseptically removed. Axillary and inguinal LN from normal mice were pooled, while from MSV tumor-bearing mice, draining right inguinal and lumbar nodes were investigated separately from nondraining left axillary nodes. Single cell suspensions were made in medium RPMI 1640, supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine, and 5% of fetal bovine serum (all reagents obtained from Grand Island Biological Co., Grand Island, N. Y.). A vol of 0.2 ml, containing 6×10^5 cells were cultured in each well of tissue culture-treated U plates (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.) at 37°C in an atmosphere of 5% CO₂ and 95% air. For determination of "spontaneous" (mitogen-independent) DNA synthesis, spleen cells were cultured for 16 h immediately after their removal with 5 µCi/ml [³H]thymidine (TdRH3) (sp act 6 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.).

Routinely, several PHA doses (Burroughs Wellcome, Beckenham, England) were tested in each experiment. Under all conditions tested here, the dose responses of MSV animals were similar to those of normal animals. Therefore, only results obtained with optimal PHA doses (1 µg/ml) are included in most of the tables. In these experiments cultures were incubated for 48 h, after which TdRH3 was added for another 16–18 h. Cultures were harvested by a multiple-automated sample harvester (Microbiological Associates, Inc., Bethesda, Md.) on glass fiber filters. These were taken up in Aquasol^R (New England Nuclear, Boston, Mass.) and counted in a liquid scintillation counter. The results are expressed as mean cpm of triplicate samples. The standard errors of this assay are 5–10% and are subsequently not included in the tables.

³ For brevity, spleens from mice carrying primary MSV-induced tumors, 12–16 days after virus inoculation will be termed MSV spleens.

Rayon Adherence Columns.—Two large size rayon balls (Parke Davis & Co., Detroit, Mich.) were packed into 12-ml syringes (Monoject, Sherwood Medical, Industries, Inc., Deland, Fla.). These were autoclaved and washed with 50 ml of balanced salt solution. About 10^8 spleen cells in 7 ml of complete RPMI 1640 medium (GIBCO) were incubated for 15–20 min on these columns at room temperature and then slowly eluted. About 20–25% of the original cell population were recovered from these columns. Preliminary investigations have demonstrated the presence of functionally intact B and T lymphocytes in the column eluates, but phagocytic cells were effectively eliminated.

Treatment of Spleen Cells with Carbonyl Iron and Magnet.—Lymphocyte separating agent, containing carbonyl iron particles, was obtained from Technicon Instruments Corp., Tarrytown, N. Y. $3\text{--}5 \times 10^7$ spleen cells were suspended in 7 ml of this reagent and incubated for 60 min at 37°C in conical 50-ml tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The tubes were then put on top of a magnet and the supernate removed by Pasteur pipettes. This procedure was repeated 6–8 times and the cells were washed once before cultivation.

Treatment by Anti- θ Serum.—Anti- θ serum was produced in AKR mice by a procedure identical to that used by Reif and Allen (16). Treatment with this antiserum plus guinea pig complement (C') has been previously shown to completely eliminate stimulation of B6 spleen cells by PHA and concanavalin A, while it did not affect the proliferative response to bacterial lipopolysaccharide (endotoxin) (LPS) (17). Spleen cells were incubated with anti- θ for 45 min at 0°C, washed, and resuspended in 1:3 diluted guinea pig C' (Texas Biological Co., Fort Worth, Texas) for 30 min at 37°C. Control spleen cell suspensions were treated with C' alone.

Treatment of Spleen Cells by Bromodeoxyuridine.—Single cell suspensions of normal or MSV spleens in RPMI 1640 (GIBCO) were prepared as usual. 5-bromo-2'-deoxyuridine (BUDR) (Schwarz/Mann Div., Becton, Dickinson & Co.) was added at a concentration of 30 $\mu\text{g}/\text{ml}$, and the cells were cultured in $16 \times 125\text{-mm}$ plastic tubes (Falcon Plastics, Div. of BioQuest) for about 6 h (total vol 3 ml, cell density $1 \times 10^6/\text{ml}$). They were then illuminated, as described by Zoschke and Bach (18), washed three times, and resuspended in medium.

^{51}Cr -Release Assay of Cellular Cytotoxicity.—The method of measuring cytotoxicity of MSV spleen cells against RBL-5 ascitic lymphoma cells, has been described in detail previously (7). Briefly, 10^7 spleen cells were incubated for 4 h with 5×10^4 ^{51}Cr -labeled target cells in $35 \times 10\text{-mm}$ plastic petri dishes on a rocking platform. The background ^{51}Cr release was determined by incubation of the ^{51}Cr -labeled target cells with unlabeled target cells. Experimental results were expressed as the percent lysis in the experimental group minus the background lysis.

Primary In vitro Immune Response to Sheep Red Blood Cells (SRBC).—The technique of Mishell and Dutton (19) was followed with minor modifications. Briefly, 10^7 spleen cells were cultured in 1.0 ml of Eagle's minimal essential medium (GIBCO) plus 10% fetal bovine serum in $35 \times 10\text{-mm}$ plastic petri dishes (Falcon Plastics, Div. of BioQuest). They were incubated for 4 days on a rocking platform in an atmosphere of 10% CO_2 , 10% O_2 , and 80% N_2 . SRBC were added at a concentration of $10^7/\text{ml}$ and after 4 days plaque-forming cells (PFC) were enumerated by the technique of Cunningham and Szenberg (20). All assays were performed in triplicate.

RESULTS

Some Characteristics of Spleens from MSV Tumor-Bearing Mice.—MSV spleens were considerably enlarged and contained three to four times as many higher numbers of mononuclear cells than spleens from normal controls (Table I). The T-cell population, as estimated by the number of cells killed by anti- θ serum plus C' was about 5–10% lower in MSV spleens than in controls. The number of phagocytic cells was increased about threefold in spleens from tumor-bearing mice. The TdRH3 uptake of MSV spleen cell cultures on a per cell

basis was increased 8–10 times when measured during the first 16 h after removal of the spleens and compared with spleens from normal B6 mice. This elevated “spontaneous” (mitogen-independent) DNA synthesis was reduced to normal values when MSV spleen cells were treated by passage through a rayon adherence column. It was reduced by 70% when MSV spleen cells were incubated with iron particles and subsequently exposed to a magnet (Table II). However, it was not reduced after treatment with anti- θ serum plus C'. The low spontaneous DNA synthesis of normal spleens was also reduced to some extent by the adherence columns and the iron magnet technique, but was not affected by anti- θ treatment.

PHA Response of Spleen and Lymph Node Cells from MSV Tumor-Bearing Mice.—The PHA response of MSV spleen cells was strongly depressed at all mitogen doses. In some experiments almost no response was seen (as in the experiment shown in Table III). In others, the response was decreased 80–90%, when compared with matched control mice (Tables IV and V). When the PHA response of pooled LN lymphocytes from normal and MSV mice were compared,

TABLE I
Some Characteristics of Spleens from B6 Mice with Primary MSV-Induced Tumors, 14 Days after Virus Inoculation

	MSV spleen	Normal spleen
Weight*	278.3 mg	73.4 mg
No. of mononuclear cells*	2.82×10^8	0.76×10^8
Percentage of macrophages§	10.2	3.5
Percentage of T cells§	25.3	32.7
DNA synthesis/ 10^6 cells	42,480 cpm	4,328 cpm

* Measured immediately after removal, mean of 10 mice.

† Determined after phagocytosis of latex particles.

§ Number of cells, killed by anti- θ serum plus C' (trypan blue exclusion test), mean of five experiments.

|| TdRH3 incorporation during the first 18 h after spleen removal (no mitogen was added), mean cpm of five experiments.

TABLE II
Effect of Various Treatments on TdRH3 Uptake of Spleen Cells from MSV Tumor-Bearing and Normal B6 Mice

Treatment before culture	MSV spleen	Normal spleen
None	22,319*	2,625
Eluate from rayon column	718	818
Iron/magnet	4,918	648
Anti- θ plus C'	18,408	2,643

* Mean cpm of triplicate cultures of 6×10^5 cells; TdRH3 incorporation during the first 18 h after spleen removal without added mitogen.

TABLE III
PHA Stimulation of Spleen and LN Lymphocytes from MSV Tumor-Bearing Mice

Source of Cells	Dose of PHA ($\mu\text{g/ml}$)			
	None	5	2.5	1.25
I				
MSV spleen	5,749*	3,848	8,271	9,115
Normal spleen	1,842	72,894	84,952	126,297
MSV pooled LN	4,968	23,174	53,294	33,843
Normal pooled LN	1,320	140,612	88,432	80,212
II				
MSV draining LN	14,507	17,412	16,596	13,842
MSV distal LN	6,603	80,504	49,730	17,826
Normal pooled LN	304	120,801	74,203	53,288

* Mean TdRH3 uptake of triplicate cultures at 72 h after a 16 h pulse of isotope, the SE of this assay is always <10% and not included in the tables.

TABLE IV
Comparison of Specific Cytotoxicity and PHA Responsiveness of Spleen Cells from B6 Mice Carrying Three Different Types of Tumors

Tumor system	PHA stimulation	
	<i>cpm</i>	%
Standard MSV	16,342	29.6
Gz-MSV(M) 14‡	12,442	22.7
Gz-MSV(M) 28‡	14,812	3.6
RBL-5§	10,837	4.1
Normal B6	98,712	2.3

* As determined by ^{51}Cr release from syngeneic ascitic RBL-5 lymphoma cells in a 4 h assay.

‡ Gz-MSV(M)-induced, progressively growing tumors 14 and 28 days after inoculation.

§ Spleens from B6 mice carrying the ascitic lymphoma RBL-5 7 days after tumor cell inoculation.

TABLE V
Restoration of PHA Reactivity of MSV Spleen Cells by Rayon Adherence Columns and Iron/Magnet Treatment

Source of cells	Treatment			
	PHA	None	Column	Iron/Magnet
MSV spleen	—	4,370*	1,314	2,623
	+	20,167	84,450	61,330
Normal B6 spleen	—	3,655	589	620
	+	92,633	107,405	69,627

* Mean cpm of triplicate cultures of 6×10^5 cells.

a defect was also observed. However, this defect was more marked when LN draining the tumor site were studied separately. The two primary draining LN, the right inguinal and lumbar nodes are usually much larger than the contralateral nodes and are easily identifiable. Lymphocytes from these were almost totally unresponsive, while cells from the distal LN revealed a PHA response closer to the reactivity of normal pooled LN lymphocytes (Table III).

Correlation Between PHA Responsiveness and Specific Cytotoxicity of MSV Spleens.—Specific cytotoxicity of MSV spleen cells against syngeneic lymphoma cells was measured in a ^{51}Cr -release assay. An inverse relationship between the development of cytotoxic effector cells in MSV spleens and the depression of the PHA response was consistently observed (Fig. 1). Cytotoxicity and PHA responsiveness were also compared in two additional tumor systems. In spleens from mice carrying the RBL-5 leukemia in ascitic form, the PHA response was strongly suppressed, and there was only low cytotoxic activity (Table IV). B6 mice, injected with Gz-MSV(M) demonstrated appreciable specific cytotoxicity only during the first 21 days. Later in the course of the progressively growing tumor, only low levels of cytotoxic activity could be demonstrated. In contrast, the PHA response was defective through the entire growth period of the tumor.

Reconstitution of the PHA Response of MSV Spleen Cells by Adherence Columns or by Iron/Magnet Treatment.—The defective PHA response of MSV spleen cells could be restored by passage of the cells through rayon adherence columns before PHA stimulation (Table V). The proportion of the total spleen cell

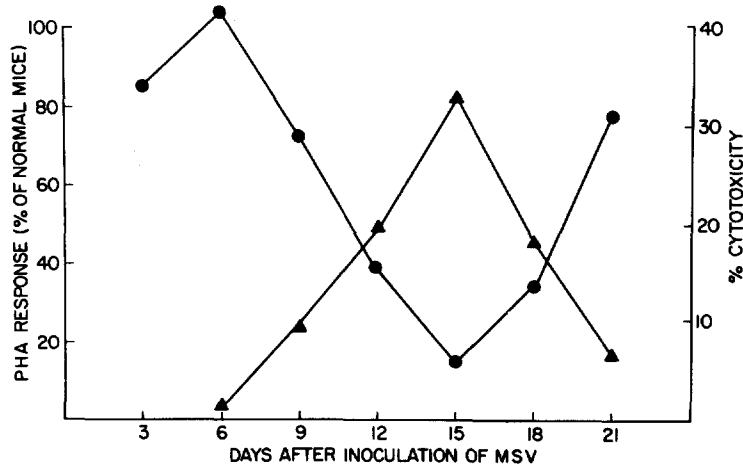


FIG. 1. Correlation between the development of specific cytotoxicity (\blacktriangle — \blacktriangle) and the depression of the PHA response (\bullet — \bullet) in spleens of B6 mice bearing primary MSV-induced tumors. Cytotoxicity was determined by the percentage ^{51}Cr release from syngeneic lymphoma cells and PHA reactivity is expressed as the percent of TdRH3 uptake of simultaneously tested normal PHA-stimulated cultures.

population removed by this type of column was similar in normal and MSV spleens (70–80%). However, column treatment caused only a small increase in TdRH3 uptake of PHA-stimulated normal spleen cells at the cell densities we have used in our experiments. Mixing of column-purified MSV spleen cells with unseparated isogenic spleen cells reduced the PHA response to the level seen before column treatment (Table VI).

Spleen cells were also incubated with carbonyl iron particles and treated by a magnet. This treatment removed about 30% of the cell population of both normal and MSV spleens. It led to an increase of PHA reactivity of MSV spleen cell cultures comparable to that observed after column treatment (Table V).

Inhibitory Effect of MSV Spleen Cells on the PHA Response of Normal Spleen Cells.—The above experiments suggested a possible role of a suppressor cell in the inhibition of the PHA response. To directly demonstrate such an activity, MSV spleen cells were mixed with syngeneic normal spleen cells before PHA was added. In these experiments the fixed number of normal spleen cells was 6×10^5 and various numbers of MSV spleen cells were added. A suppressive effect on the PHA response of normal spleen cells could be demonstrated when 2×10^5 or more MSV spleen cells were added (Table VII). Admixture of additional normal spleen cells had no inhibitory effect. Similarly, spleen cells from

TABLE VI

Mixing of Column-Purified MSV Spleen Cells with Autologous-Unpurified Spleen Cells

	No mitogen	PHA
A Unpurified MSV spleen	4,841	8,920
B Column-purified MSV spleen	3,152	110,001
C A and B at a 1:1 ratio	5,582	10,004
D Unpurified normal spleen	2,820	88,412
E Column-purified normal spleen	1,213	106,412
F D and E at a 1:1 ratio	1,923	112,418

TABLE VII

Inhibitory Effect of MSV Spleen Cells on the PHA Response of Spleen Cells from Syngeneic Normal Mice

Source and no. of Cells	No mitogen	PHA
6×10^5 normal spleen cells	1,354	77,321
6×10^5 MSV spleen cells	2,870	18,342
6×10^5 normal and 4×10^5 MSV	3,330	24,330
6×10^5 normal and 2×10^5 MSV	3,140	29,340
10×10^5 normal spleen cells	2,640	104,328
8×10^5 normal spleen cells	2,349	93,480

Before being placed in culture various numbers of MSV spleen cells or normal spleen cells were added to a fixed number of normal spleen cells (6×10^5).

B6 mice after regression of MSV tumors were ineffective. The suppressor activity of MSV spleen cells was eliminated after passage through adherence columns and treatment by the iron/magnet technique (Table VIII). It was unaffected by treatment with anti- θ plus C'.

Effect of Pretreatment by BUDR on the PHA Response of MSV Spleen Cells.—A comparison between the experiments of Table II and Table VIII showed that the techniques which remove cells synthesizing DNA from MSV spleens, also abolished the suppressive capacity of this population, suggesting that the suppressor cell is a proliferating cell. Further evidence for this assumption was obtained by pretreatment of MSV spleen cells with BUDR, which has been recently shown to selectively inactivate DNA-synthesizing cells in cultures of lymphoid cells (18). MSV spleen cells were treated with BUDR for 6 h and extensively washed before addition of PHA. In this experiment, BUDR-treated MSV spleen cells demonstrated a significantly higher (about threefold) TdRH3 uptake after PHA stimulation than did cultures of untreated MSV spleen cells (Table IX). In contrast, preincubation with BUDR caused no increase of PHA reactivity of normal spleen cells.

Lack of Suppressive Activity by Spleen Cells from Nude Mice and Spleen Cells Treated with Anti- θ Serum.—Spleen cells from nude mice, shown to be totally unresponsive to PHA, were added to normal Balb/C spleen cells before addition of PHA. These nude mice had gone through several backcross generations

TABLE VIII

Effect of Various Treatments of MSV Spleen Cells on Their Ability to Suppress the PHA Response of Normal Spleen Cells

Treatment*	Normal spleen cells	Normal and MSV spleen cells
None	93,831	16,378
Anti θ and C'	—	18,744
Rayon column	—	84,314
Iron/magnet	—	68,412

* 3×10^5 MSV spleen cells, treated by various methods were added to 6×10^5 normal B6 spleen cells before the addition of PHA.

TABLE IX

Effect of Pretreatment with BUDR on the PHA Response of Normal and MSV Spleen Cells

Source of spleen cells	BUDR	No mitogen	PHA
MSV	—	6,340	22,112
	+	1,814	63,120
Normal	—	1,733	78,214
	+	833	72,319

Spleen cells were incubated for 6 h with or without 30 $\mu\text{g/ml}$ of BUDR, illuminated and washed several times before the addition of PHA.

with Balb/C, but were not syngeneic. This accounts for the observed low grade mixed leukocyte reaction (Table X). The PHA response of Balb/C spleen cells remained unaltered after addition of nude spleen cells. Normal B6 spleen cells, unreactive to PHA after treatment with anti- θ plus C' were also ineffective in inhibiting the PHA response of syngeneic untreated spleen cells (Table XI). These two experiments show that it requires more than just dilution by PHA unreactive spleen cells to inhibit the PHA response of normally reactive spleen cells.

Primary In Vitro Immune Response of MSV Spleen Cells to SRBC.—To test another lymphocyte function in addition to the PHA response, the primary in vitro antibody response of MSV spleen cells to SRBC was also investigated. After 4 days in culture spleen cells from tumor-bearing mice produced less than 10% the number of PFC produced by normal controls (Table XII). This

TABLE X
Effect of Spleen Cells from Nude Mice on the PHA Response of Balb/C Spleen Cells

Source of cells	No mitogen	PHA
6×10^5 Balb/C spleen cells	1,843	136,321
6×10^5 nude spleen cells	2,241	2,333
6×10^5 Balb/C and 3×10^5 nude spleen cells	7,347*	129,453

* Nude mice had been backcrossed with Balb/C mice but were not syngeneic, a mixed leukocyte reaction was therefore observed.

TABLE XI
Effect of Anti- θ -Treated Spleen Cells on the PHA Response of Syngeneic B6 Spleen Cells

Cells added to 6×10^5 B6 spleen cells	No mitogen	PHA
None	1,120	86,315
4×10^5 spleen cells treated by C' alone	2,370	115,368
4×10^5 spleen cells treated with anti- θ and C'	3,839	89,348
6×10^5 C'-treated spleen cells alone	2,241	121,340
6×10^5 anti- θ and C'-treated cells alone	1,873	2,245

TABLE XII
In Vitro Response to SRBC of Spleen Lymphocytes from Normal B6 and MSV Tumor-Bearing Mice

Source of cells	SRBC	Plaque-forming cells per culture
Normal spleen	—	71 (32-253)*
	+	4,051 (3,376-4,871)
MSV spleen	—	11 (3-39)
	+	57 (18-178)

* Geometric means and 67% confidence limits of four pooled experiments.

defect could not be restored by the addition of 2-mercaptoethanol (data not shown). The recovery of viable cells in normal controls was about 40%, while in MSV spleen cells it was about 30%. This difference in cell survival cannot account for the inability of MSV spleen cells to mount an immune response to SRBC, since at a survival rate of 30% the normal spleen cells still respond almost normally.

DISCUSSION

There is considerable controversy as to whether the presence of a tumor leads to depression or stimulation of the immune system. Splens and draining LN of mice with methylcholanthrene-induced transplanted tumors show morphological signs of activation and elevated DNA synthesis (12, 21). With the use of discontinuous albumin density gradients, changes in these spleens have been demonstrated, similar to those seen after immunization with SRBC (12). In contrast, numerous studies (for review see references 4 and 5) have shown marked deficiencies in the cellular immune response of tumor-bearing patients and of experimental animals. Signs of splenic activation, similar to those described above, were seen in our study using B6 mice carrying primary MSV-induced tumors. These spleens were considerably enlarged, contained four times the normal number of mononuclear cells, and displayed markedly elevated DNA synthesis on a per cell basis. Similar observations were also made in LN draining the site of the MSV tumor.

However, the PHA responses of these spleens and LN on a per cell basis were strongly suppressed. This defect was reversed by adherence column treatment, and by treatment with iron plus magnet, a technique which mainly removes phagocytic cells. There are several possible explanations for the reconstituting effects, obtained by these techniques: (a) PHA reactive T cells in MSV spleens are simply diluted out by cells unreactive to PHA; (b) T lymphocytes of MSV spleens, rendered unreactive to PHA are altered by these techniques in a way that restores their PHA reactivity; and (c) Suppressor cells are present in MSV spleens, which suppress the *in vitro* PHA response, and these cells are removed by the column or by the iron/magnet treatment.

All the data we have obtained in this study point toward the third of these possibilities. The activity of suppressor cells was directly demonstrated when MSV spleen cells were mixed with normal spleen cells before addition of PHA. Spleen cells from nude mice and syngeneic normal spleen cells treated by anti- θ and C', both totally unreactive to PHA, had no inhibitory effect. This demonstrates that it requires more than just dilution by PHA unresponsive cells to suppress the PHA response of normal spleen cells.

The concept of suppressor cells regulating the immune response has been recently emphasized. Several types of suppressor cells have been described. Thymic-derived suppressor cells (22-26) have been thought to regulate B-cell activity in a feedback mechanism. Suppressor cells which act on the *in vitro*

proliferation of T cells have also been described. These have included peritoneal and alveolar macrophages (27), splenic macrophages (28), and adherent spleen cells, thought to be different from macrophages (29). The suppressor cell of the PHA response, which we have found in spleens of MSV tumor-bearing mice, has the following characteristics: It was not inactivated by treatment with anti- θ plus C' and therefore appears to be not a T lymphocyte. Since the suppressor cell was removed by the adherence columns and the iron/magnet technique, it seems to be a cell of the monocyte/macrophage series. However, the magnet technique in our hands removes a larger percentage of spleen cells than are phagocytic by morphological criteria. It is therefore not possible to completely rule out the possibility that the suppressor cell is for example an adherent subpopulation of B cells.

We have found that the same techniques that abolished the suppressive effect of MSV spleen cells, also greatly reduced their elevated "spontaneous" (mitogen-independent) DNA synthesis (compare Table II and VIII). It is therefore possible that the cells with elevated DNA synthesis and the suppressor cells of the PHA response are identical. This was also suggested by our findings that BUDR treatment, before addition of PHA, resulted in a significant increase of PHA reactivity of MSV spleen cells. A technique, selecting for cells in the DNA-synthesizing phase of the cell cycle (30) may therefore be suited to enrich suppressor cells.

An inverse relationship was observed between the development of specific cytotoxic effector cells and the development of suppressor cells in the standard MSV system. Therefore, one may ask whether the cytotoxic effector cells and the suppressor cells are identical. However, the effector cell in the ^{51}Cr -release assay of cytotoxicity has been previously shown to be a T cell (31, 32), while the suppressor cell of the PHA response appears not to be a T cell. Furthermore, in related tumor systems (Table IV) a strong depression of the PHA response was seen in the absence of significant cytotoxicity.

The mechanisms that cause the development of suppressor cells in tumor-bearing mice are as yet not clearly understood. However, the depression of the PHA response in MSV spleens clearly paralleled the development of the tumor and was no longer demonstrable after tumor regression (8). It therefore seems likely that the tumor itself releases substances that are transported to the spleen with resultant activation of suppressor cells. The suppressor cells seem to have a somewhat selective effect on T-lymphocyte functions, since we have recently shown that the response of MSV spleen cells to concanavalin A, another T-cell mitogen (33), was depressed in a similar manner as the PHA response (8). In contrast, the responses to LPS and pokeweed mitogen, both known to stimulate murine B lymphocytes (33, 34), were affected less. In spleens of mice carrying the RBL-5 lymphoma in ascitic form, this was even more marked, since we have observed completely normal responses to LPS, at a time when PHA reactivity was maximally suppressed.¹ In the present

investigation we have also found that the primary *in vitro* response to SRBC was totally abolished in spleens of mice with MSV tumors. This function is known to depend on a cooperation between B and T cells (35), and in analogy with previous findings in another tumor system (36) one may assume that the defect observed in the MSV spleens is also primarily a defect of the helper T cell.

Gorczyński has presented evidence that lymphocyte stimulation by tumor-associated antigens was similarly affected as the PHA response by suppressor cells (14). Tumor-associated immunity, measured by ^{51}Cr release from syngeneic tumor cells, seems to be unaffected by these suppressor cells. Column purification which removed the suppressor cells of the PHA response, did not increase but moderately decreased the activity of MSV spleen cells in the Cr-release assay of specific cytotoxicity.⁴ Furthermore, studies now in progress have shown that mitogen-induced cytotoxicity and lymphocyte-induced antibody-dependent cytotoxicity are normal in MSV spleen cells.⁵ Obviously, the suppressor cell we have described only affects certain aspects of lymphocyte reactivity, and further work is necessary to clarify the nature of this selectivity.

There are striking parallels between our results in a tumor system and a recent report (28) on the effects of infection with *Corynebacterium parvum* and of a graft-vs.-host reaction. In both cases markedly depressed PHA responses were observed and activation of suppressor cells, supposed to be macrophages, could be demonstrated. In another study of spleen cells undergoing a graft-vs.-host reaction (37), a depression of the primary *in vitro* antibody response to SRBC by suppressor cells was found, again paralleling our results in a virus-induced tumor system. The results of Gorczyński (14) and our study suggest that the activation of suppressor cells is an important feature in the alterations of the immune system induced by a growing tumor. Tumors, as repeatedly observed, induce a strong activation of the reticuloendothelial system. However, during this activation, activation of suppressor cells also occurs, which then interferes with selected aspects of the immune response. It is conceivable that these suppressor cells also interfere with the immune reaction against the tumor itself. This hypothesis, however, needs further experimentation for support.

SUMMARY

Spleens from Moloney sarcoma virus (MSV) tumor-bearing C57BL/6N mice contained four times the normal number of mononuclear cells and displayed a markedly elevated "spontaneous" (mitogen-independent) DNA synthesis on a per cell basis. The number of macrophages were increased three-fold while there was a slight reduction in the percentage of T lymphocytes. The phytohemagglutinin (PHA) response on a per cell basis of spleens from

⁴ H. T. Holden and H. Kirchner, unpublished observations.

⁵ A. Muchmore and H. Kirchner, unpublished observations.

tumor-bearing mice was decreased about 90% when compared with normal control mice. The primary *in vitro* immune response to sheep red blood cells was also suppressed to levels of less than 10% of normals.

The PHA response could be restored by purification of MSV spleen cells by rayon adherence columns and by removal of phagocytic cells by an iron/magnet technique. The activity of suppressor cells in MSV spleens was demonstrated in mixtures with syngeneic normal spleen cells where a marked impairment of the PHA response was observed. Spleen cells from tumor-free nude mice and normal spleen cells treated by anti- θ serum plus guinea pig complement (C'), both totally unreactive to PHA, had no such effect. The inhibitor cell in MSV spleens was shown to be insensitive to inactivation by anti- θ plus C', but could be removed by the adherence columns and the iron/magnet technique. These data suggest that this suppressor cell is a cell of the monocyte/macrophage series. Suggestive evidence was also presented that the suppressor cells belong to a proliferating population in MSV spleens. Similar suppressor cells have been previously demonstrated in spleens of mice during a variety of immune responses. Our data show, that a tumor, although stimulating the immune system, nevertheless may be suppressive on certain immune functions through the activation of suppressor cells.

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REFERENCES

1. Burnet, F. M. 1957. Cancer—a biological approach. III. Viruses associated with neoplastic conditions. *Br. Med. J.* **1**:841.
2. Smith, R. T., and M. Landy, editors. 1970. *In Immune Surveillance*. Academic Press, Inc. New York.
3. Klein, G. 1969. Experimental studies in tumor immunology. *Fed. Proc.* **28**:1739.
4. Smith, R. T. 1972. Possibilities and problems of immunological intervention in cancer. *N. Engl. J. Med.* **287**:439.
5. Herberman, R. B. 1974. Cell mediated immunity to tumor cells. *Adv. Cancer Res.* In press.
6. Hellström, K. E., and I. Hellström. 1974. Lymphocyte mediated cytotoxicity and blocking serum activity to tumor antigens. *Adv. Immunol.* In press.
7. Lavrin, D. H., M. Nunn, N. Soares, and R. B. Herberman. 1973. *In vitro* cytotoxicity studies of murine sarcoma virus (MSV)-induced immunity in mice. *J. Natl. Cancer Inst.* **51**:1497.
8. Kirchner, H., R. B. Herberman, M. Glaser, and D. H. Lavrin. 1974. Suppression of *in vitro* lymphocyte stimulation in mice bearing primary Moloney sarcoma virus-induced tumors. *Cell. Immunol.* In press.
9. Hersh, E. M., and J. J. Oppenheim. 1965. Impaired *in vitro* lymphocyte transformation in Hodgkin's disease. *N. Engl. J. Med.* **273**:1006.
10. Garrioch, D. B., R. A. Good, and R. A. Gatti. 1970. Lymphocyte response to PHA in patients with non-lymphoid tumors. *Lancet.* **1**:618.
11. Sample, W. F., H. R. Gertner, and P. B. Chretien. 1971. Inhibition of phyto-

- hemagglutinin-induced *in vitro* lymphocyte transformation by serum from patients with carcinoma. *J. Natl. Cancer Inst.* **46**:1291.
12. Adler, W. H., T. Takiguchi, and R. T. Smith. 1971. Phytohemagglutinin unresponsiveness in mouse spleen cells induced by methylcholanthrene sarcomas. *Cancer Res.* **31**:864.
 13. Häyri, P., D. Rago, and V. Defendi. 1970. Inhibition of phytohemagglutinin and alloantigen-induced lymphocyte stimulation by Rauscher leukemia virus. *J. Natl. Cancer Inst.* **44**:1311.
 14. Gorczynski, R. M. 1974. Immunity to murine sarcoma virus induced tumors. II. Suppression of T cell-mediated immunity by cells from progressor animals. *J. Immunol.* In press.
 15. McCoy, J. L., A. Fefer, and G. P. Glynn. 1967. Comparative studies on the induction of transplantation resistance in Balb/C and C57Bl/6 mice in three murine leukemia systems. *Cancer Res.* **27**:1743.
 16. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med.* **120**:413.
 17. Rosenstreich, D. L., A. Nowotny, T. Chused, and S. R. Mergenhagen. 1973. *In vitro* transformation of mouse bone marrow derived (B) lymphocytes induced by the lipid component of endotoxin. *Infect. Immun.* **8**:406.
 18. Zoschke, D. C., and F. H. Bach. 1970. Specificity of antigen recognition by human lymphocytes *in vitro*. *Science Wash. D. C.* **170**:1404.
 19. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
 20. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody forming cells. *Immunology.* **14**:599.
 21. Risdall, R. J., J. C. Aust, and C. F. McKhann. 1973. Immune capacity and response to antigenic tumors. *Cancer Res.* **33**:2078.
 22. Allison, A. C., A. M. Denman, and R. D. Barnes. 1971. Cooperating and controlling functions of thymus derived lymphocytes in relation to autoimmunity. *Lancet.* **2**:135.
 23. Droege, W. 1971. Amplifying and suppressive effect of thymus cells. *Nature (Lond.)*. **234**:549.
 24. Baker, P. J., P. W. Stashak, D. R. Amsbaugh, and R. F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumopolysaccharide. *J. Immunol.* **105**:1581.
 25. Gershon, R. K., P. Cohen, R. Hencin, and S. A. Liebhaber. 1972. Suppressor T cells. *J. Immunol.* **108**:586.
 26. Chused, T. M., A. D. Steinberg, and L. M. Parker. 1973. Enhanced antibody response of mice to polyinosinic polycytidylic acid by antithymocyte serum and its age-dependent loss in NZB/W mice. *J. Immunol.* **111**:52.
 27. Parkhouse, R. M. E., and R. W. Dutton. 1966. Inhibition of spleen cell DNA synthesis by autologous macrophages. *J. Immunol.* **97**:663.
 28. Scott, M. T. 1972. Biological effects of the adjuvant *Corynebacterium parvum*. I. Inhibition of PHA, mixed lymphocyte and GVH reactivity. *Cell. Immunol.* **5**:459.
 29. Folch, H., M. Yoshinaga, and B. H. Waksman. 1973. Regulation of lymphocyte responses *in vitro*. III. Inhibition by adherent cells of the T-lymphocyte response to phytohemagglutinin. *J. Immunol.* **110**:835.

30. Everson, L. K., D. N. Buell, and G. N. Rogentine. 1973. Separation of human lymphoid cells into G1, S, and G2 cell cycle populations by use of a velocity sedimentation technique. *J. Exp. Med.* **137**:343.
31. LeClerc, J. C., E. Gomard, F. Plata, and J. P. Levy. 1973. Cell mediated reactions against tumors induced by oncornaviruses. II. Nature of the cells which are involved in tumor cell cytotoxicity. *Int. J. Cancer.* **11**:426.
32. Herberman, R. B., M. E. Nunn, D. H. Lavrin, and R. Asofsky. 1973. Effect of antibody to θ antigen on cell mediated immunity induced in syngeneic mice by murine sarcoma virus. *J. Natl. Cancer Inst.* **51**:1509.
33. Anderson, J., G. Möller, and O. Sjöberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell. Immunol.* **4**:381.
34. Janossy, G., and M. F. Greaves. 1971. Lymphocyte activation. I. Response of B and T lymphocytes to phytohemagglutinin. *Clin. Exp. Immunol.* **9**:483.
35. Vann, D. C., and J. R. Kettman. 1972. *In vitro* cooperation of cells of bone marrow and thymus origin in the generation of antibody-forming cells. *J. Immunol.* **108**:73.
36. Rowland, G. F., A. J. Edwards, M. R. Summer, and C. M. Hurd. 1973. Thymic dependency of tumor-induced immunosuppression. *J. Natl. Cancer Inst.* **50**:1329.
37. Sjöberg, O. 1972. Effect of allogeneic cell interaction on the primary immune response *in vitro*. Cell types involved in suppression and stimulation of antibody synthesis. *Clin. Exp. Immunol.* **12**:365.