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Suppressive and Cytostatic Activities in the Spleen of Tumor-Bearing Hamsters*

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Abstract—*Tumors were induced in hamsters by injection of two different tumor cell types: EHB, a spontaneously transformed cell line, which displayed non-antigenicity by all immunological techniques tested. The second cell line used was a SV_{40} transformed hamster fibroblast (TSV_5 Cl_2) which possesses all known SV_{40} induced antigens. Spleen cells of TBH were tested for their ability:* (1) *to suppress stimulation of normal spleen cells by Con A and* (2) *to inhibit multiplication of tumor cells* in vitro.

In EHB tumor bearing hamsters the two activities appeared when the tumor had reached about 2 g *and persisted until the death of the animals. In (TSV_5Cl_2) TBH the two activities appeared earlier (tumor weight* 0.5–1 g) *and disappeared when the tumor was bigger than* 7–9 g. *The cells responsible for both activities were not T-cells, were adherent and destroyed by silica: a good candidate might be the macrophage. The two activities were found in parallel and depend on the type of tumor cells used and the size of the tumor.*

INTRODUCTION

SEVERAL investigators have demonstrated the presence of cells able to suppress the mitogen-induced stimulation of normal lymphocytes in the spleen of tumor bearing animals. Suppressor cells participate in a number of critical immunologic phenomena of immunologic tolerance and antigenic competition. Some suppressor regulatory functions are antigen specific, whereas others are non specific. Suppressor cells may play an important role in tumor immunology, may interfere in the immune response against the tumor graft and may prevent tumor rejection by the host.

Suppressor cells are observed, during tumor growth, in tumor-bearing mice [1, 2], rats [3, 4] and humans [5]. They are characterized as T-cells for the some [6–8] and 'not-T' cells for others [3, 9. 10].

Suppressor cells have been described in virally induced tumor systems after the tumors had reached a considerable size. As there was no data available supporting the existence of suppressor cells during the early development of virus-induced tumors, we chose to study the suppressive activity of spleen cells from tumor bearing hamsters. Tumors were induced by SV_{40} transformed and strongly antigenic cells (TSV_5Cl_2) or by spontaneously transformed and non-antigenic cells (EHB). The two systems were compared and the non-specific suppression expressed by spleen cells from tumor bearing hamsters (TBH) was tested using Con A as mitogen since we had shown previously that hamster spleen lymphocytes respond poorly to LPS [11, 12].

Attempts were made to identify indirectly the suppressive cells by testing activity after different pretreatments of the spleen cell suspensions. These *in vitro* studies demonstrate that suppressor cells were of a not T-cell origin.

In parallel, spleen cell suspensions were tested for their ability to inhibit tumor growth *in vitro*. Cytostasis was measured by the inhibition of nucleotide incorporation by tumor cells. Cells involved in this activity are not T-cells and not tumor specific as described by Kirchner *et al.* [13] and Lespinats and Poupon [14].

MATERIALS AND METHODS

Animals

Syrian hamsters, 5–6 months old maintained in a closed colony in the Institut de Recherches Scientifiques sur le Cancer (Villejuif, France) were used.

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Cell lines

The following cell lines were used: TSV_5Cl_2; this line arose from an undifferentiated tumor appearing in a Syrian hamster injected at birth with 10^5 PFU of SV_{40} virus and maintained in culture after cloning (Tournier *et al.*, 1967). EHB: spontaneously transformed hamster fibroblasts.

Tumors

Two types of tumors were used: Tumor EHB; induced in the hamster by injection of 5×10^4 spontaneously-transformed cells. Tumor TSV_5Cl_2; induced by injection of 5×10^4 cells transformed by the simian-virus 40 (SV_{40}) (Tournier *et al.* 1967).

Tumors appeared about 3 weeks after injection and the hamsters died 8–9 weeks after the tumor graft.

Antisera against hamster IgG and hamster thymocytes

The rabbit antiserum against hamster IgG (RaHIgG) and the rabbit serum against hamster thymocytes (RaHThy) has been described (de Vaux Saint Cyr *et al.*, 1977).

The RaHIgG was used at 1:250 dilution and the RaHThy at 1:200 dilution in the presence of guinea pig complement (Institut Pasteur, Paris).

Mitogen

Concanavalin A (Con A), (Calbiochem, England) was added to lymphocyte cultures at a dose of 2 μg/ml, found to be optimal for stimulation of NspL [12].

Spleen cell suspension preparation

Spleens were aseptically removed, minced with scissors, filtered through a 200-mesh stainless steel sieve (Tamisor), and washed twice with RPMI 1640 (Eurobio-France). They were resuspended in RPMI 1640 supplemented with 2 mM glutamine (Gibco, Grand Island, N.Y.), penicillin 100 U/ml, streptomycin 100 μg/ml and foetal calf serum 5% at the appropriate cell concentration after counting the viable cells by the trypan blue exclusion method. Viability was usually greater than 95%.

Lymphocyte culture

The degree of stimulation was assessed by measuring incorporation of tritiated thymidine into mitogen-treated cells, Aliquots of 100 μl containing various numbers (3, 6 or 9×10^5) of spleen cells from normal (NSpC) or spleen cells from TBH (TBH SpC) were placed in the wells of Falcon 3040 microplates (Falcon plastics, Oxnard, Calif.) and to each well were added successively: (a) 100 μl of either nutrient medium, or 100 μl of a second suspension of lymphoid cells; (b) 50 μl of either nutrient medium of 50 μl of medium containing the mitogen (2 μg/ml of Con A).

All cultures were carried out in triplicate. The plates were covered and incubated at 37°C in a humid 5% CO_2 + 95% air atmosphere for 48 hr. Five hr before the end of the culture 1 μCi of (^{3}H) TdR (TMN 48 Centre de l'Energie Atomique, Gif-sur-Yvette, France) was added to each well. Cultures were harvested with a Multiple Automated Sample Harvester (Microbiological Associates Inc. Bethesda) on glass fiber (Reeve Angel, Clifton, N.Y.). The glass fiber discs containing the radioactive cells were placed in toluene Omnifluor (New England Nuclear Corp. DreiechenHainm, Federal Republic of Germany) and the radioactivity was counted in an Intertechnique Tricarb Scintillator.

Results are expressed as mean counts/min of triplicate ±S.E. The suppressor effects of population of spleen cells from TB hamsters (TBH SpC) upon stimulation of the spleen cells from normal animals (NSpC) was calculated by the following formula:

$$100 - 100 \times \frac{\text{counts/min (TBHSpC + NSpC) stimulated} - \text{counts/min (TBHSpC + NSpC) unstimulated}}{\text{counts/min (NSpC) stimulated} - \text{counts/min (NSpC) unstimulated}}$$

Cytotoxicity tests

Fifty μm aliquots of a suspension of NSpC or TBHSpC (4×10^6/ml) were placed in 5 ml plastic tubes and 100 μl of either RaHIgG (1:250) or RaHThy (1:200) antiserum was added. After 20 min incubation at 0°C, 100 μl of absorbed guinea complement diluted 1:6 was added. Following a further 40 min at 37°C, the cells were stained with trypan blue, counted, and adjusted to the desired concentration.

Silica treatment

Silica cytotoxicity for mouse macrophages in culture was demonstrated by Marks (1957). Ten μl of a suspension containing 2 mg/ml of silica was added to each well, and tests were performed as described above.

Separation of SpC by Falcon adherence

The total SpC population was incubated in Falcon Petri dishes for 1 hr at 37°C in 5% CO_2 atmosphere. The non-adherent cells were then removed, counted, and tested for their suppressor effect. The cells adherent to the petri dish were rinsed twice with medium, and trypsin was added. The cells were collected by scraping with a rubber policeman, centrifuged, counted and tested as described above.

Cytostatic assay

The TSV_5Cl_2 and EHB cells are cell lines maintained *in vitro* since 1967 (Tournier *et al.*). After trypsinization the cells were washed resuspended in RPMI-1640 medium and adjusted at 6×10^4 cells/ml. One hundred μl were seeded in each well of Falcon 3040 microplates (Falcon Plastics, Oxnard, Calif.) and incubated overnight at 37°C in 5% CO_2.

Spleen cells were routinely used at three effector cell:target cell ratios 25:1, 50:1 and 100:1. The medium was removed from the tumor cells in the microplates, aliquots of 100 μl containing the spleen cells were added to the wells and to each well was added a further 100 μl of nutrient medium. The plates were covered and incubated at 37°C in an atmosphere of 5% CO_2 for 48 hr after which 1 μCi of (3HTdR) was added to each well for 5 hr. Cultures were harvested with a MASH apparatus and radioactivity counted. The results are expressed in counts/min as mean counts of triplicate samples ±S.E. The inhibition was calculated as:

$$100 - 100 \times \frac{\text{counts/min in test}}{\text{counts/min in control}}$$

Controls were target cells with normal spleen cells at the same ratio as in the test.

RESULTS

Changes in the spleen of TBH

Normal spleens of 5-month old hamsters yield an average of 35×10^6 nucleated cells. In TBH, spleen weight and lymphocyte counts increased considerably during the growth of the tumor as shown in Fig. 1. The splenomegaly was greater in (EHB) TBH than in (Cl_1) TBH.

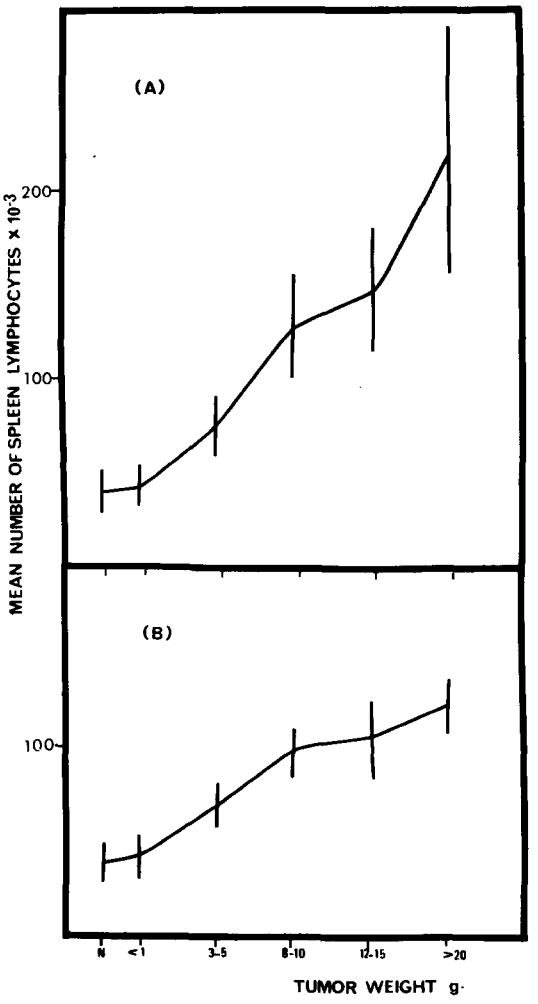

Fig. 1. Mean number of spleen lymphocytes as a function of tumor weight. (A): (EHB) tumor bearing hamsters, (B): (TSV_5Cl_2) tumor bearing hamsters.

The Con A response of SpC from normal and TB hamsters

Stimulation by Con A is age related in hamsters [12]. The NSpC of 2-month old animals responded very strongly to simulation by mitogens but old hamsters responded poorly. In both cases the inhibition by SpC from TBH was difficult to ascertain. For this reason 5-month old hamsters were chosen throughout the experiment. In a previous paper we have shown [11, 12] that the hamsters from our Institute animal farm responded well to stimulation by Con A and poorly to PHA which are both T-cell mitogens. Con A was chosen as a T-cell mitogen for hamster SpL. The Con A response of SpC from TBH was depressed as compared to that of SpC from normal controls. These experiments were reproducible and the results of a representative experiment are presented in Table 1. The depression

Table 1. Response to Con A of TBH SpC as a function of tumor weight

Tumor weight	Counts/min 6.10^5 TBH SpC*	6.10^5NSpC	ΔCounts/min†
0.5 g	209,255 ± 22,457	162,428 ± 13,022	−46,827
2.5 g	78,182 ± 10,640	230,979 ± 16,005	152,797
6.5 g	97,352 ± 3284	158,320 ± 7201	60,968
9.0 g	103,401 ± 9760	247,806 ± 11,908	144,405
25 g	99,911 ± 5055	177,669 ± 10,376	77,758

*SpC from (EHB) tumor bearing hamsters.
†ΔCounts/min = counts/min (NSpC + Con A) − counts/min (TBH SpC + Con A).

appeared always for a tumor weight greater than 2 g.

Inhibitory effect of SpC from TB hamsters on the mitogen response

SpC from TBH were collected at different intervals during the growth of the tumor. At the ratio of 2 TBHSpC: 1 NSpC they strongly inhibit the Con A stimulation of normal SpC in both systems. The SpC from hamsters bearing a tumor induced by SV_{40} transformed cells (Fig. 2) showed a suppressive effect as soon as the tumors reached about 1 g and nearly no suppressive effect could be detected when the tumor weight had exceeded to 10–15 g. The maximum of the inhibition reached 50–60% and was found in animals bearing tumors weighing 4–7 g.

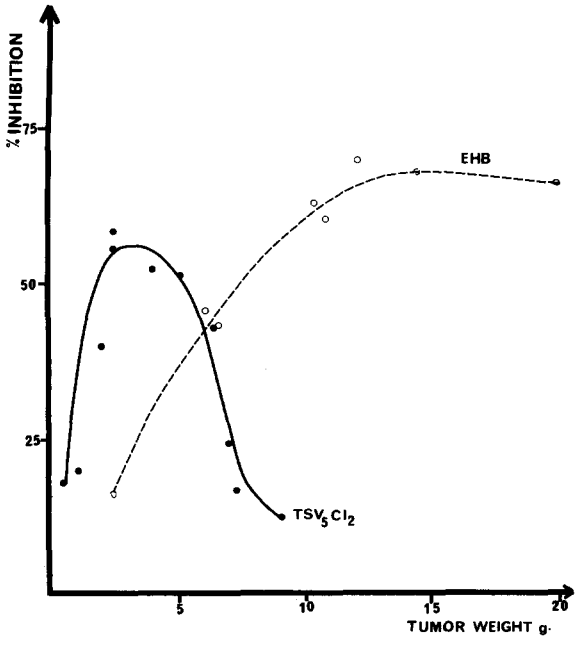

Fig. 2. Suppressive activity of the TBH SpC as a function of tumor weight, inhibition of the NspC response to Con A (2 μg/ml) by: (-----) (EHB) TBH SpC, (————) (TSV_5Cl_2) TBH SpC.

In spleen cells of (EHB) TB hamsters the suppressive activity appeared later, at a tumor weight of about 5 g (Fig. 2) and persisted throughout tumor development until the death of the animal. The Con A response of NSpC decreased 55–65% for tumor weights of 12–17 g and the percentage of inhibition reached a plateau thereafter.

Con A stimulation as a function of lymphoid cell concentration per well was tested. As anticipated the Con A response 9×10^5 NSpC was considerably higher than that of 3×10^5 NSpC (Table 2). Therefore the decrease of the Con A stimulation in the mixture of 3×10^5 NSpC + 6×10^5 TBHSpC was not due to cell crowding (Table 2).

Table 2. Con A stimulation as a function of concentration of normal spleen

NSpC	Mit	Counts/min ± S.E.
3×10^5	+	80,340 ± 12,301
6×10^5	+	230,309 + 26,153
9×10^5	+	248,688 ± 5081

Effect of pretreatment of TBHSpC on suppressive activity

(1) *Treatment of cells by adherence technique.* In the two tumoral systems, removal of cells by adherence to Falcon completely abolished the suppressive effect (Table 3). Non-adherent cells were strongly stimulated by Con A; this fraction enriched in T-cells, showed no suppressive effect of the stimulation of NSpC by Con A. The adherent fraction, consisting mainly of macrophages and B cells when harvested from Petri dishes, showed a strong suppressive effect on NSpC.

(2) *Treatment with silica.* Treatment of TBHS with Silica, known to be cytotoxic for

Table 3. Effect of the treatment of TBH spleen cells on the suppressive activity

NSpC	TBHSpC* (6×10^5)	Mit	Counts/min ± S.E.	Suppressive effect (%)
3×10^5	Whole population	+	44,514 ± 12,123	46
3×10^5	Non-adherent population†	+	98,918 ± 7004	0
3×10^5	Adherent population†	+	50,512 ± 10,316	38
3×10^5	Treated by silica	+	98,902 ± 12,108	0
3×10^5	Treated by RaHThy + C	+	52,023 ± 8311	35
3×10^5	Treated by RaHIgG + C	+	69,971 ± 5915	14

*Spleen lymphocytes from 2 (EHB) TBH with tumor weights between 5 and 6 g.
†Non-adherent cell population and adherent cell population from 6×10^5 TBH spleen cells.

mouse macrophages, completely abolished their suppressive activity on NSpC.

(3) *Pretreatment of TBHSpC with antisera + C′.* After treatnent of TBHSpC with RaHThy + C′, no difference was observed as suppressive activity remained. After treatment with RaHIgG + C′ the suppressive activity of TBHSpC was abolished in part. These results indicate that cells responsible for the suppressive effect in tumor bearing hamsters are adherent, killed by silica and are lysed in part by an antiserum against immunoglobulin plus complement. Macrophage found in TBH spleens which may have membrane bound IgG on their surface are the most probable candidates for displaying the suppressive activity on the stimulation of NSpC by Con A.

Cytostatic activity

As shown in Fig. 3, spleen cells from TB hamsters, exerted a strong inhibitory effect on (^{3}H) thymidine incorporation by cultured tumor cells. This inhibition could be detected at effector: tumor cell ratios as low as 25:1, but the best ratio was 100:1. This ratio was used throughout all the tests with NSpC and TBHSpC. Tumor cells used were EHB and TSV_5Cl_2 cells. The (^{3}H) thymidine incorporation of the spleen cells alone was very low about 0.3–1% of the incorporation of tumor cells and was therefore neglected. The NSpC had an inhibitory effect on the growth of tumor cells of about 40–50% and the TBHSpC exerted an ever stronger inhibition on the incorporation of (^{3}H) thymidine of tumor cells. The differences between the numbers obtained with NSpC and those obtained with TBHSpC in parallel experiments were considered as cytostatic activity. The high inhibitory effect of NSpC may be due to natural killer cells (Haddada *et al.*, in preparation).

The kinetics of the cytostatic activity of SpC of (EHB) TBH and of (TSV_5Cl_2) TBH were very similar to the curves obtained when suppressive actibity was tested in these two tumoral systems. The cytostatic activity depended on the type and the size of the tumor; it appeared earlier and was easier to detect than the suppressive activity.

In (TSV_5Cl_2) TBH this activity appeared earlier than the suppressive one, showed a maximum for tumor weights between 3 and 7 g, decreased abruptly and showed a residual cytostatic activity of 15%. In (EHB) TBH this activity could be detected for tumors weighing 2 g and increased with the tumor growth until the death of the animal.

Pretreatment of TBHSpC by Falcon adher-

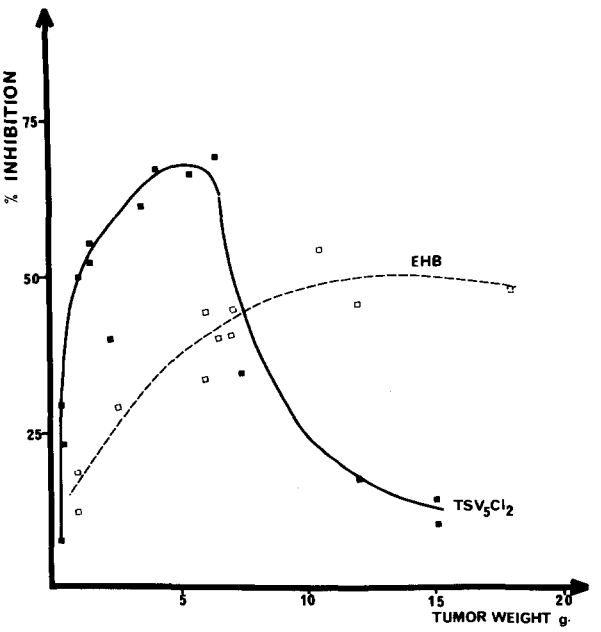

Fig. 3. The cytostatic activity as function of tumor weight. Inhibition of the tumor cell multiplication in vitro *by:* (- - - - -) *(EHB) THB SpC,* (————) *(TSV_5Cl_2) TBH SpC, at the ratio of:* 100 *SpC:* 1 *tumor cell.*

ence technique and removal of adherent cells decreased the inhibitory effect.

Two types of tumor cells were used, TSV_5Cl_2 and EHB: SpC from hamsters bearing the one or the other type of tumor were equally active on both cell lines demonstrating that the cytostatic activity was non-specific.

DISCUSSION

Suppressor cells have been extensively studied in mouse [1, 2, 10] and man [5] and shown to participate in a number of critical immunologic phenomena and antigenic competition. Certain classes of B-cells, T-cells and macrophages are thought to play roles in suppression. Some suppressive functions appear to be antigen specific whereas other are non-specific. Kilburn *et al.* [15] and Kirchner *et al.* [9], have demonstrated suppressor cells in spleens of mice bearing progressive tumors. These suppressor cells inhibit lymphocyte stimulation by mitomycin C-treated lymphoma cells. Poupon *et al.* [10] have shown the presence of suppressor cells in spleens of mice bearing methylcholanthrene induced tumors. These three groups showed that the cells responsible for tumor suppression are not T-cells. Gorczyinski concluded that they are B-cells, Kirchner *et al.* and Lespinats *et al.* believe them to be macrophages. In the two systems described here a non-specific suppressive effect and a cytostatic activity which seem be due to the same adherent cells were demonstrated in tumor bearing hamsters.

TSV_5Cl_2 and EHB cells are both hamster fibroblasts but EHB is a spontaneously transformed cell line which, by all immunological techniques tested, displays no antigenicity whatsoever. The suppressive and cytostatic activities found in the SpL of (EHB) TBH are very comparable to those found in SpL of methylcholanthrene (MC) induced tumors by Lespinats *et al.* These MC mice tumors are poorly immunogenic and no circulating antibodies directed against membrane associated antigens can be demonstrated exactly as in the EHB tumor system studied here. On the other hand, TSV_5Cl_2 cells are SV_{40} transformed fibroblasts which possess all recognized SV_{40} coded antigens both nuclear and associated with the cell membranes. De Vaux Saint Cyr *et al.* [16] have shown that early after the appearance of the tumor the presence of circulating antibodies as well as antibody containing cells in the peritumoral area and in the spleen. Furthermore, the antibody titer in the sera of hamsters bearing TSV_5Cl_2 increases as a function of tumor growth. It is of interest that the suppressive and cytostatic activities present in SpC of (TSV_5Cl_2) TBH are very different from that seen in (EHB) TBH. In the case of TSV_5Cl_2, both activities appear when tumors have reached about 1 g, show a maximum for weights between 3 and 7 g, and decrease thereafter quite abruptly. In the EHB tumors the level of inhibition remains elevated until the death of the hamster. The suppressive and cytostatic activities disappear in (TSV_5Cl_2) TBH at about the time that circulating antibodies become detectable in the sera [16]. One question remains unanswered: is there any necessary relationship between the presence in the spleen of antibody forming cells and the absence of suppressor cells?

As the same spleen cell populations were tested for their ability to inhibit both the multiplication of tumor cells (cytostasis) and mitogen-induced lymphocyte stimulation (suppression) one can postulate that the same 'not-T' and adherent cell is the one which bears the two activities. One strong argument which favors this hypothesis is the striking similarity between the curves of cytostasis and of suppression. The cells responsible for the suppressive activity are 'not-T' lymphocytes, adherent and destroyed by silica. Cells responsible for cytostatic activity are also 'not-T' and adherent cells: a good candidate may be the macrophage.

Several authors [17–19] attribute the immunosuppressive effect to humoral factors, or to macrophage-derived soluble factors that inhibit mitogen-induced lymphocyte blastogenesis [20] and contact sensitivity reactions [21]. Soluble immune complexes that activiate T suppressor cells have also been proposed [22], soluble tumor associated-antigens have been shown to activate suppressor cells and to induce the formation of humoral blocking factors [23]. The occurrence of suppressor macrophage does not seem to be restricted to the tumor situation. For example, they have also been found in spleens of mice undergoing a graft vs host reaction and in mice injected with *Corynebacterium parvum* [24]. It has also been pointed out that the depression of mitogen reactivity by macrophages in spleen cultures may simply reflect the increased number of macrophages present in spleens of tumor bearing animals [25]. It may also be that suppressor cells comprise a particular subclass of macrophages or constitute cells in a particular state of activation, but such a

formulation will not account for our results. It seems also that in the TSV_5Cl_2 tumor system, the suppressive effect of macrophages is without effect on the *in vitro* antibody response directed against SV_{40} induced antigens early in the tumor growth. The disappearance of the suppressive activity after the TSV_5Cl_2 tumor has reached a weight of 8–10 g and the presence of this activity throughout growth of the EHB tumor suggest that the appearance of suppressor macrophages is not the result of stimulation by immunogenic tumors.

The cytostatic effect is due to cells which are adherent. On many occasions, the same spleen cell populations were tested for their ability to inhibit both multiplication of tumor cells and Con A induced lymphocyte stimulation. Both effects were found in parallel, and the cells responsible for these effects were 'not-T', adherent cells. The high level of inhibition of ^3H TdR incorporation in tumor cells by NSpC may be explained by the presence of natural killer or natural cytotoxic cells detected by the chromium release test in the spleens of normal hamsters (Haddada *et al.*, in preparation).

In conclusion, it can be said that spleens of TB hamsters contain cells which have a non specific inhibitory effect on the proliferation of cells having a high rate of multiplication and that they act *in vitro* on tumor cell growth and lymphoid cell division.

The presence of these cells seems to depend on the immunogenicity of the tumor cells injected in animals being more evident in the case of non-immunogenic tumors both in hamsters as shown here and in mice as shown by Lespinats [14].

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