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In Vitro Inhibition of Lymphoproliferative Responses to Tumor Associated Antigens and of Lymphoma Cell Proliferation by Rat Splenic Macrophages

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Spleens from W/Fu rats bearing a syngeneic progressively growing (C58NT)D tumor contain cells which can inhibit lymphoproliferative responses in a mixed lymphocyte-tumor interaction designed to demonstrate suppressor activity. Spleens from rats having rejected (C58NT)D tumors also contained suppressor cells but to a lesser degree. The growth inhibition assay, which measures inhibition of proliferation of tumor cells, was evaluated as a simple assay system to screen for suppressor cell activity. The effector cells in both assays had the same characteristics, indicating a predominant role of macrophages. Normal rat spleens were found to contain growth inhibition activity which led to the demonstration of suppressor cell activity in spleens of normal animals. Removal of suppressor cells from the spleens of immunne rats results in consistently higher lymphoproliferative responses to tumor associated antigens on the tumor cells.

INTRODUCTION

Lymphoproliferative responses and responses dependent upon them have been somewhat difficult to demonstrate with rat spleen cell preparations (1). Only recently has the *in vitro* generation of a primary cytotoxic response to alloantigen, using rat spleen cells, been demonstrated (2). Frequent demonstration of strong proliferative responses using other rat lymphoid populations suggests that the defect in the spleen might be due to the presence of inhibitor cells rather than to an inherent lack of cells capable of response. Folch and Waksman (3) have reported suppressor cell activity in the spleens of normal rats responsible for inhibition of proliferative responses. Glaser *et al.* (4) have described suppressor cells in the spleens of rats bearing a Gross virus-induced lymphoma, (C58NT)D, which inhibit T cell responses to mitogens and to tumor associated antigens. In tumor-bearing mice, suppressor macrophages have been demonstrated to be active in inhibition of not only mitogen-stimulated lymphocytes, but also of rapidly proliferating tumor cells growing in tissue culture (5, 6).

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In the present investigation, we have studied in more detail the nature of the suppressor cells in the spleens of rats bearing the (C58NT)D tumor. We have also examined the possibility that, as in the mouse (5, 6), similar or identical effector cells can inhibit *in vitro* proliferation of both lymphocytes and tumor cells. The use of the growth inhibition assay with tumor cells to predict suppressor cell activity in various lymphoid populations is demonstrated.

MATERIALS AND METHODS

Animals

Inbred W/Fu rats were obtained from Microbiological Associates, Walkersville, Md.

Tumors

The W/Fu rat lymphoma (C58NT)D (7), induced by Gross leukemia virus, was maintained in ascites form in weanling male W/Fu rats by serial intraperitoneal passage of 2×10^7 viable cells.

French (C58NT)D (see below) was maintained in a similar way.

(C58NT)D tissue culture has been described (8).

LW-12 is a methylcholanthrene-induced granulocytic leukemia in W/Fu rats. An established culture line of LW-12 (9) was obtained from Dr. Stuart Aaronson, NCI.

RBL-5 is a Raucher virus-induced leukemia syngeneic to C57BL/6 mice and has been fully described elsewhere (10). An established culture line of RBL-5 was obtained from Dr. Howard T. Holden, NCI.

An established culture line of L1210, a DBA/2 mouse lymphoma, was kindly provided by Dr. Stephan Haskill, Medical University of South Carolina.

All of the tissue cultured cells were mtaintained in RPMI 1640 with 20% FBS. The cultures were supplemented daily with fresh medium and they were split when necessary to keep the cells in a rapid phase of growth.

Immunizations

Regressor system: 1×10^8 (C58NT)D tumor cells were inoculated subcutaneously in the right flank of 8-week-old male W/Fu rats. This inoculation resulted in transient tumor growth with tumors appearing about Days 6–7, reaching a peak size on Days 10–12, and usually regressing by Days 14–15 (11).

Progressor system: 1×10^8 French NTD tumor cells (a variant of (C58NT)D recently obtained from Dr. J. Dore, Villejuif, France) was injected subcutaneously in the right flank of 8-week-old male W/Fu rats. This inoculation resulted in progressive tumor growth, locally palpable at Days 4–5 and usually associated with ipsilateral lymph node involvement by Days 10–12, and ultimately ending with the death of the animal with widespread metastases 30–40 days after inoculation.

Preparation of Lymphoid Cells

Spleen, lymph node and thymus cell suspensions were prepared as previously described (11). Spleen cells obtained from regressors or progressors are designated as Reg- or Pro-, followed by the number of days after tumor cell inoculation (e.g., Reg-30, for spleen cells from regressors, 30 days after inoculation).

Cells Separation Procedures

Unless otherwise stated, the medium used for all experiments and cell separation procedures was as follows: medium 1640 (Grand Island Biological Co.) containing 25×10^{-3} *M* Hepes solution (Microbiological Associates, Inc., Bethesda, Md.), 5×10^{-5} *M* 2-mercaptoethanol (2-ME) (Schwarz-Mann Division, Becton, Dickinson & Co., Orangeburg, N.Y.), 100 units of penicillin per ml, 100 μg of streptomycin per ml, 2 m*M* of glutamine and 10% FBS (all reagents from Grand Island Biological Co., Grand Island, N.Y.). This will be referred to as complete medium.

Carbonyl iron and magnet. 1×10^8 spleen cells in 2 ml of medium were combined with 200 mg of carbonyl iron powder (GAF Corp., New York, N.Y.) and incubated for 1 hr at 37°C in a shaking water bath. Following the incubation period, the cell suspension was diluted to 5 ml and serially poured from test tube to test tube using a strong magnet to retain cells having phagocytized the iron powder at each step. A series of 6–8 test tubes was used. This procedure resulted in a decrease from 8–10% to 1–2% in the proportion of normal spleen cells which could ingest latex particles.

Passage of spleen cells through a rayon column. Thirty-five milliliter syringes were disassembled, and the barrel was filled with three rayon cotton balls (Parke Davis Co., Detroit, Mich.). The assembly was then autoclaved and prepared for use as follows: A sterile stopcock was placed on the tip of the syringe and 50 ml of Hanks' balanced salt solution (BSS) were rapidly passed through the column. Then $5–8 \times 10^8$ spleen cells in media were placed on the column and incubated at 37°C in a 5% CO_2 humid atmosphere. After 20 min incubation, the nonadherent cells were eluted with 10 ml of warm (37°C) BSS, centrifuged at 250 g for 10 min, and resuspended in complete medium. Twenty to 30% of the cells were recovered by this procedure. Latex particle ingesting cells decreased from 8–10% of the normal spleen population to 1–2%, and with spleens from animals bearing tumors, decreased from 13–15% to 1–3%. This procedure had virtually no effect on the percentage of cells bearing surface immunoglobulin (40–50% of normal spleen population as measured by membrane surface immunofluorescence with fluorescein conjugated goat anti-rat immunoglobulin (Lot No. 4020201, prepared for NCI by Huntington Research Center, Brooklandville, Md.). The levels of cell-mediated cytotoxicity of immune spleen cells against (C58NT)D, which is T-cell dependent in this system (12), were either unchanged or increased after column passage.

Removal of T cells. A rabbit anti-rat thymocyte serum (ATS) was kindly provided by Drs. B. Veit and J. Feldman of the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. The method for producing this antiserum has been described (13). One-tenth milliliter of this antiserum (diluted 1:7) and 0.1 ml of undiluted fresh guinea pig serum as a source of complement were added to 0.1 ml of 1×10^7 spleen cells, and the mixture was incubated 37°C for 1 hr. The cells were then washed twice with 50 ml of BSS and resuspended in complete medium. This procedure consistently and completely abrogated the proliferative response of the spleen cells to PHA and the cytotoxicity of immune lymphocytes against (C58NT)D.

Removal of cells bearing surface immunoglobulin. One-tenth of a milliliter of undiluted goat anti-rat gamma globulin (Lot 8554, Cappel Laboratories, Inc., Downingtown, Pa.) was added to 1×10^7 spleen cells and incubated at 22°C for

15 min. Then 0.1 ml of undiluted guinea pig complement was added and the mixture was incubated at 37°C in a moist 5% $C°O_2$ atmosphere for 60 min. The cells were then washed three times with BSS. This procedure routinely decreased the number of cells bearing surface immunoglobulin from 40–50% in the normal spleen population to approximately 7%, as determined by surface immunofluorescence (described above).

Treatment of cells with carrageenan. Carrageenan (Seakem 9; RE-7059) was a gift of MCI Biomedical (Division of Maine Colloids, Inc., Rockland, Maine). Spleen cells were incubated in complete medium containing 400 mg/ml of carrageenan for 4 hr at 37°C. The cells were then washed twice with BSS and resuspended in complete medium. With similar treatment, carrageenan has been shown to inactivate macrophages without affecting T and B cell function (5, 14).

Assays of Lymphoid Cell Functional Activity

The mixed lymphocyte tumor interaction (MLTI) with C58NT)D was performed as previously described (15) except that 2-ME was present in the medium. The addition of 2-ME has been shown to significantly improve lymphocyte proliferative responses in the rat, without altering the kinetics or specificity of the responses (16). Briefly, 2×10^4 (C58NT)D tumor cells treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) were cocultivated with 8×10^5 immune or normal spleen cells in 0.2 ml of medium for 96 hr in the wells of tissue culture-treated U plates (Cook Engineering Co., Alexandria, Va.).

One microcurie of [^{3}H]thymidine {([^{3}H]TdR) specific activity 6 Ci/mmol; Schwarz-Mann, Orangeburg, N.Y.; final concentration 5 μCi/ml} was added to each well for the final 16 hr of the incubation period, and the cultures were harvested and further processed as previously described (5). All cultures were carried out in triplicate. All standard errors were less than 5% and are not included in the tables.

Suppressor cell activity was demonstrated as described previously (4). Briefly, various numbers of mitomycin C-treated spleen cells from the populations being tested for suppression were added to a fixed number of rayon column passaged immune spleen cells (8×10^5/well). MLTI was then done as above.

The method for *in vitro* generation of secondary cell-mediated cytotoxicity has been described in detail (17). Briefly, 2×10^7 spleen cells were cultivated in 5 ml of complete medium in 30 ml tissue culture flasks (Falcon Plastics, Oxnard, Ca.) in the upright position at 37° for five days either alone or with 5×10^5 mitomycin C-treated tumor cells. After incubation, the cells were washed once and assayed for cytotoxic activity as described below.

The method for ^{51}Cr release cytotoxicity assay has been described in detail (18). Briefly, 1×10^4 ^{51}Cr-labeled (C58NT)D tissue culture target cells were incubated with 5×10^5 effector spleen cells on a rocking platform at 37°C in a humid 5% CO_2 atmosphere for 4 hr. The percent cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{cpm } ^{51}\text{Cr released from cells in the experimental group} - \text{cpm } ^{51}\text{Cr released in the control group}}{\text{cpm } ^{51}\text{Cr in cells at initiation of assay}} \times 100$$

The control group consisted of labeled tumor cells in media alone. All standard errors were below 5% and are not shown in the results.

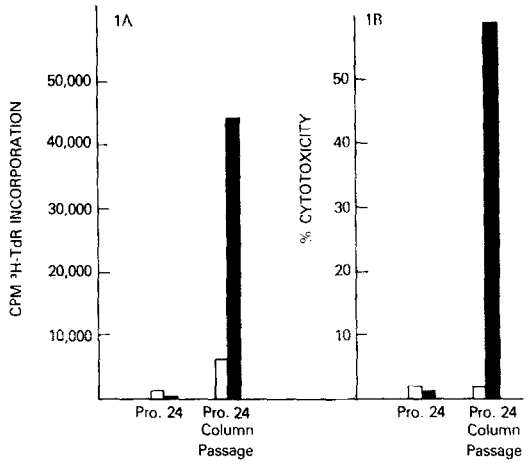

FIG. 1A. Effect of depletion of adherent cells on MLTI. Open bars represent spontaneous proliferation of spleen cells from rats 24 days after inoculation with French NTD, cultured in medium alone. Shaded bars represent the proliferative response to mitomycin C-treated (C58-NT)D cells. 1B. Effect of depletion of adherent cells on the *in vitro* generation of a secondary cytotoxic response. Cells tested are from the same cultures shown in Fig. 1A. Tests for ^{51}Cr releact cytotoxicity against (C58NT)D were performed after 5 days of incubation in medium alone. Shaded bars represent lymphocytes incubated with tumor cells.

The growth inhibition assay (GIA) was modified from that of Kirchner *et al.* (6). Briefly, varying numbers of effectors cells, from 8×10^5 to 1×10^5, giving effector/target cell ratios (E/T ratios) of 40:1, 20:1, 10:1, and 5:1 were incubated at 37° in a moist 5% CO_2 atmosphere in the wells of tissue culture-treated U plates with 2×10^4 tumor tissue culture target cells in complete medium. The culture period was 48 hr with [^{3}H]TdR (sp. act. 6 Ci/mmole; final concentration 5 mCi/ml) present for the last 4 hr. Results in the GIA were expressed as percent growth inhibition (GI):

$$\% \text{ GI} = \frac{[^3\text{H}]\text{TdR incorporation of tissue culture alone} - [^3\text{H}]\text{TdR incorporation of mixture of tissue culture and effector cells}}{[^3\text{H}]\text{TdR incorporation of tissue culture alone}} \times 100$$

Standard errors of results were less than 5% and are not shown in the tables.

RESULTS

Suppression of Response to Tumor Associated Antigens in Tumor-Bearing Rats

As previously observed (4), spleen cells from animals bearing progressively growing tumors (progressor spleens) could proliferate in the MLTI only after depletion of phagocytic or adherent cells. An example of such experiments is shown in Fig. 1A.

It was of interest to determine whether the suppressor cells in rats bearing progressively growing tumors would also inhibit the *in vitro* generation of a secondary cytotoxic response against (C58NT)D. Figure 1B shows such an experiment in which a secondary response was sought in suspensions of spleen cells from animals bearing progressively growing tumors. No cytotoxicity was generated with

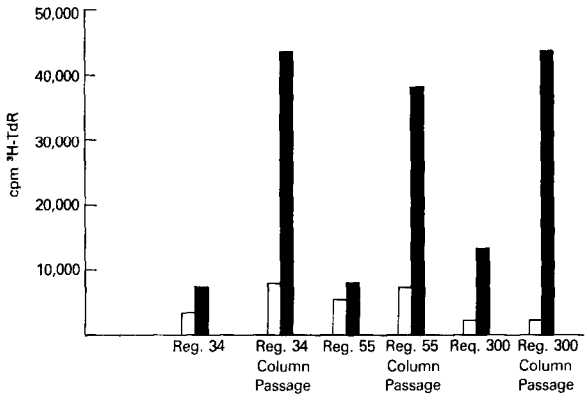

FIG. 2. Effect of depletion of adherent cells on MLTI. Shaded bars represent proliferative response of immune spleen cells from rats at various times after inoculation with (C58NT)D, after culture with tumor cells for 4 days. Open bars represent [^{3}H]Tdr incorporation of the spleen cells in medium alone.

the unfractionated spleen cells. With adherent cells depleted, however, a strong cytotoxic response was seen.

Suppressor Cell Activity in Animals Having Rejected Tumors (Regressors)

Lymphoproliferative responses in MLTI were initially described with rats which had already rejected the (C58NT)D tumor (15). However, the proliferative responses of regressors in MLTI were frequently of a lower magnitude than those of spleens of progressor animals with suppressor cells depleted. This suggested that suppressor cells also might be present in the spleens of regressor animals. A series of MLTIs were done on various spleen cell preparations, with and without depletion of adherent or phagocytic cells. Figures 2 and 3 illustrate the findings. It is clear from these experiments that depletion of adherent or phagocytic cells from the responding populations allowed much greater proliferative responses,

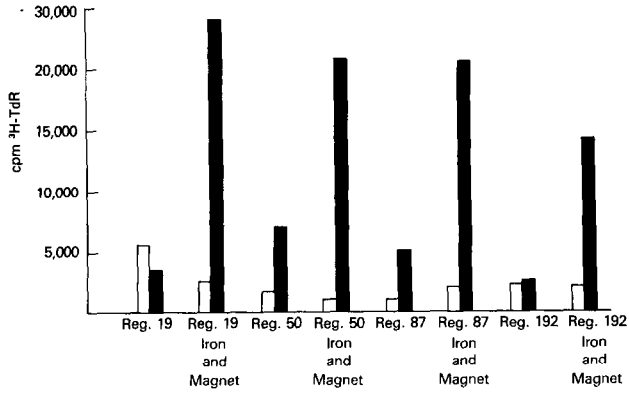

FIG. 3. Effect of depletion of phagocytic cells on MLTI. Shaded bars represent the proliferative response of immune spleen cells from rats at various times after inoculation with (C58-NT)D, after culture with tumor cells for 4 days. Open bars represent [^{3}H]Tdr incorporation of the spleen cells in medium alone.

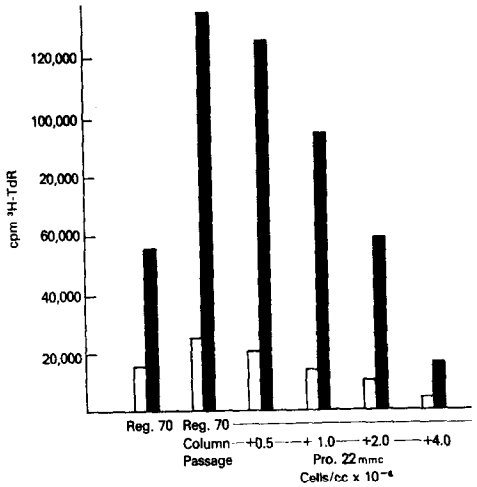

FIG. 4. Effect of adding increasing numbers of mitomycin C-treated progressor spleen cells into MLTI cultures of column passaged Reg-70 cells plus (C58NT)D. Shaded bars represent proliferative response after culture of spleen cells with tumor cells. Open bars represent proliferation of spleen cells cultured in medium alone.

which were then comparable to the responses obtained with adherent cell-depleted progressor spleen.

To directly demonstrate that the effects of column passage or carbonyl iron treatment were related to removal of suppressor cells, spleen populations, depleted of adherent cells, were combined with varying numbers of mitomycin C-treated progressor spleen cells. Pretreatment of progressor spleen cells with mitomycin C eliminated their ability to respond in MLTI but had only a minor deleterious effect on suppressor cell activity. As controls, progressor spleen cells were depleted of adherent cells and then treated with mitomycin C. These various lymphoid

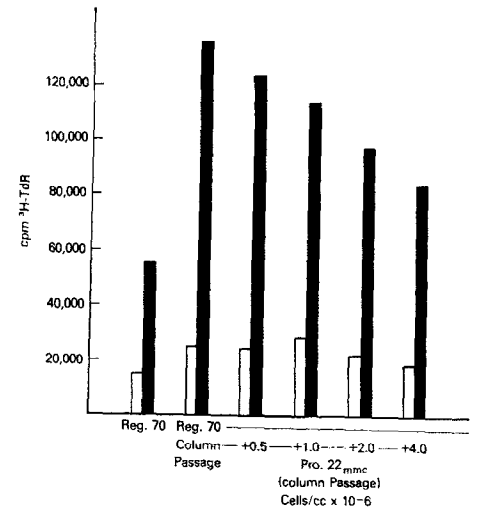

FIG. 5. The cells and experimental design here are the same as in Fig. 4, except that progressor spleen cells were passed through a rayon column prior to addition as a third party to MLTI.

populations and mixtures were then tested for responses in MLTI. The results are shown in Figs. 4 and 5. Increasing numbers of the progressor spleen cells produced an increasing degree of inhibition of response, with little detectable response when 4×10^6 progressor cells were added (Fig. 4). In contrast, after rayon column passage of the progressor cells, only minor inhibition of proliferation was seen (Fig. 5). This inhibition was probably due either to residual suppressor cells remaining in the third party population or to cell crowding and depletion of nutrients in the media.

Specificity of the Enhanced Response

Having demonstrated the effect of depleting adherent cells on the proliferative response of regressor spleen cells, it was important to be certain that the enhanced MLTI response was a specific reflection of the immune state. Experiments were therefore performed to determine whether passage of cells from normal uninoculated rats through a rayon column would enable them to react in MLTI (Table 1). In Experiment 1, the response of spleen cells of an animal one year after inoculation with (C58NT)D was augmented by depletion of adherent cells, whereas column passage of the normal spleen cells did not result in an increased rsponse above the media control. In Experiment 2, the specificity of the enhanced MLTI response was examined by performing the MLTI with LW-12 (another tumor syngeneic to W/Fu rats) as well as with (C58NT)D. The negative results with LW-12 in this experiment and the requirement for immune spleen cells demonstrated in Experiment 1 establish that after the depletion of the suppressor cells, the MLTI in the (C58NT)D system is still specific and dependent upon the presence of immune cells.

TABLE 1

Effect of Rayon Column Passage on Proliferative Response of Immune and Normal Spleen Cells in MLTI

Stimulating cells	Responding cells			
	Normal spleen	Normal spleen column passage	Reg-365	Reg-365 Column passage
Experiment 1				
Media control	6,262[a]	18,538	24,315	26,033
(C58NT)D	6,992	13,672	28,042	52,846
	Reg-100	Reg-100 Column passage		
Experiment 2				
Media control	5,830	11,640		
(C58NT)D	24,750	34,210		
LW-12	5,520	9,390		

[a] cpm [^{3}H]TdR incorporation.

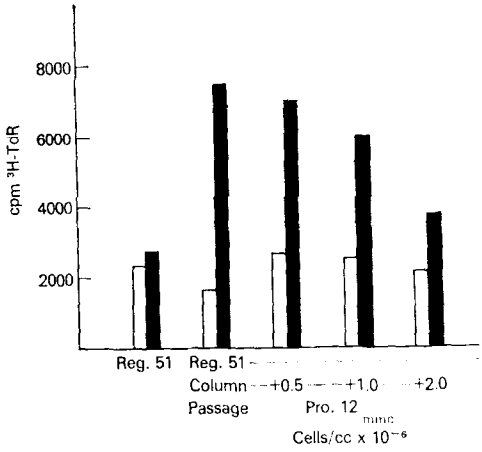

FIG. 6. MLTI with addition of progressor spleen cells done for comparison with GIA (Fig. 7). The same pool of progressor spleen cells were used in both experiments. For code see Fig. 4.

The Use of the GIA as a Test for Suppressor Cell Function

The above experiments illustrated that lymphoproliferative responses in MLTI were significantly affected by suppressor cell activity. However, the third party experiments represented a difficult assay system for suppressor cells. It was therefore of interest to develop a direct assay of suppressor cell, independent of lymphoproliferative responses. Kirchner *et al.* (5) have suggested that suppressor cells in tumor-bearing mice or in mice inoculated with *Corynebacterium parvum* also could strongly inhibit *in vitro* proliferation of tumor cells in the GIA. The effector cells in this study appeared to be of the macrophage-monocyte series, with adherent and phagocytic properties similar to those we observed with the rat suppressor cells. We therefore initiated a series of experiments to determine whether the GIA could be used as a measure of suppressor cell activity in the rat. We compared the effect of suppressor cells in the MLTI to the effect of the same cell population on tumor cells in the GIA (Figs. 6 and 7). In this experiment, Reg-51 cells were depleted of

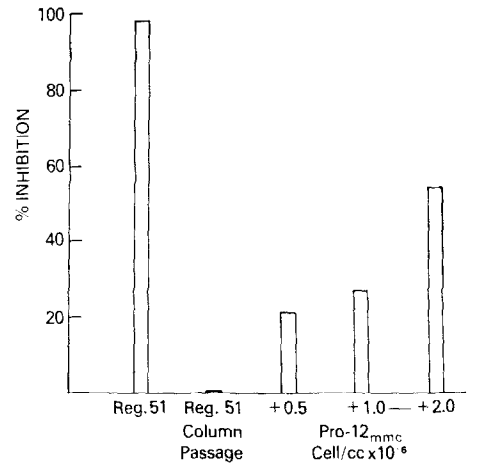

FIG. 7. Growth inhibition assay demonstrating the effect of adding increasing numbers of progressor spleen cells to immune spleen cells depleted of adherent cells. For comparison with Fig. 6.

TABLE 2

Growth Inhibitory Activity of Various Lymphoid Populations

Source of effector cells	E/T ratio	Target cell		
		(C58NT)D	L1210	RBL/5
Normal spleen	40:1	84[a]	85	86
	29:1	44	54	70
	10:1	−7	9	19
	5:1	−21	3	−5
Normal lymph node	40:1	−39		
	20:1	−51		
	10:1	−35		
	5:1	−30		
Normal thymus	40:1	−57		
	20:1	−67		
	10:1	−44		
	5:1	−27		
Pro-21 spleen	40:1	89	93	89
	20:1	73	78	82
	10:1	37	50	58
	5:1	−15	15	20

[a] Percent growth inhibition.

adherent cells and combined with varying numbers of Pro-12 spleen cells as third party suppressor cells (Fig. 6). Of note, the unfractionated spleen population (Reg-51) had a poor proliferative response which might lead one to make the erroneous conclusion that this population contained few cells capable of responding in MLTI. The spleen cells after column passage, however, showed a strong proliferative response in MLTI which decreased with the addition of increasing numbers of suppressor cells. In the GIA, the progressor spleen cell population also had a dose responsive inhibitory effect on the proliferation of the (C58NT)D tissue culture cells (Fig. 7), and the amount of inhibition of proliferation of the rapidly dividing tumor cells corresponded well with the degree of suppression observed in the MLTI. The results of this experiment are consistent with the interpretation that suppressor cell activity could be demonstrated in the GIA. However, before the GIA was used as a measure of suppressor cell activity, it was necessary to further document that the two activities were measures of the same phenomenon, i.e., that they had the same characteristics and were mediated by the same type of effector cell.

The Growth Inhibition Assay

Various lymphoid tissues were tested for growth inhibitory activity. Table 2 shows the GI effects of spleen, lymph node, and thymus of a normal animal, and of the spleen of an animal bearing a progressively growing tumor. Three antigenically unrelated tumor target cells were used. This experiment showed that normal spleen but not thymus or lymph node had growth inhibitory activity. In fact, the other lymphoid cells stimulated the incorporation of [^{3}H]TdR by the tumor cells. The spleen cells from the animal bearing a progressively growing tumor (progressor) had a greater amount of GI-activity with significant effects at 10:1 spleen cell: tumor cell ratio (E/T ratio), and this has been a consistent observation.

TABLE 3

Kinetics of Growth Inhibitory Activity in Relation to Tumor Growth in the Regressor System

Source of effector cell	E/T ratio			
	40:1	20:1	10:1	5:1
Experiment 1				
Normal Spleen	85[a]	55	47	21
Reg-6	97	72	56	47
Reg-10	96	85	71	58
Reg-14	84	65	60	34
Reg-24	89	66	59	16
Experiment 2				
Normal Spleen	94	84	52	64
Reg-13	99	97	90	47
Reg-52	96	90	67	27
Reg-89	95	87	56	8
Reg-96	94	85	54	18

[a] Percent growth inhibition of (C58NT)D tissue culture cells.

Specificity of GI-Activity

Table 2 also examines the specificity of the GIA, by use of three antigenically disparate tumor lines as target cells. Similar activity was seen against all three lines. The apparent lack of specificity of the growth inhibitory cell is consistent with the expectation that the GIA measures the effect of a cell which can inhibit a wide variety of proliferating cells, including stimulated lymphocytes. This broad reactivity is in contrast to the specificity of the cytotoxiciy of (C58NT)D immune cells previously described with the ^{51}Cr release assay (19).

Kinetics of the Growth Inhibitory Activity in Relation to Tumor Growth

Numerous reports have described a close association of suppressor cell activity with tumor growth. If the GIA measures suppressor cell activity, the kinetics also would be expected to correlate with tumor growth. Animals injected with (C58-NT)D (regressor system) or with French (C58NT)D (progressor system) were tested for GIA at various times during growth of the tumor. In Table 3, Experiment 1 shows the GI-activity of cells obtained on various days after inoculation with (C58NT)D. This experiment shows a distinct rise in activity on Days 6 and 10 with considerable activity still seen at a 5 : 1 E/T ratio. By 24 days, the level of activity had almost returned to that of the uninoculated rat. Experiment 2 further indicates that after regression of the tumor, the level of GI-activity is no higher than that seen in normal rats. It should be noted that the levels of reactivity of normal spleen cells can vary from experiment to experiment, which appears to be due to differences in the rate of proliferation of the target cell. For this reason, conclusions about relative degrees of GI-activity in various lymphoid populations had to be drawn from a single test. In each experiment, the reactivity of various populations was compared to that of a normal spleen population.

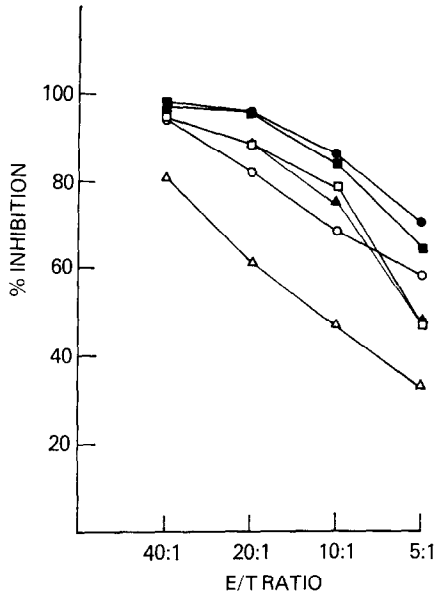

FIG. 8. Growth inhibition assay measuring relative amounts of GI-activity at various E/T ratios of spleen cells from normal rats and from rats at various times after inoculation of French NTD. △ = normal spleen, ■ = Pro-5, □ = Pro-9, ○ = Pro-11, ▲ = Pro-15, and ● = Pro-21.

The GI-activity observed in animals injected with French NTD (progressor tumor) is illustrated in Fig. 8. From this representative experiment, it is clear that the kinetics of the GI-activity were similar to that observed after inoculation

TABLE 4

Characteristics of Growth Inhibitory Cell in Normal Rat Spleen

Treatment	E/T ratios				
	40:1	30:1	20:1	10:1	5:1
Experiment 1					
Untreated	82[a]		47	1	−8
Column passage	1		−5	−13	−1
Experiment 2					
Untreated	83		41	9	0
1000 R	92		55	7	−13
2500 R	91		42	−2	−2
5000 R	83		26	−2	−21
Experiment 3					
Untreated		70			
Carrageenan		20			
Experiment 4					
Untreated		93			
Iron and magnet		−5			
Experiment 5					
Untreated	52		16	19	−2
Anti Ig and C	68		44	26	7
ATS and C	98		73	30	16
C alone	52		19	8	2

[a] Percent growth inhibition.

TABLE 5

Characteristics of Growth Inhibitory Cell in the Spleen of Animals Bearing Progressively Growing Tumors (Pro-22)

Treatment	E/T ratio			
	30:1	20:1	10:1	5:1
Experiment 1				
Untreated		75[a]	46	13
Iron and magnet		6	6	14
Experiment 2				
Untreated		39		
ATS and C		78		
ATS and C and column passage		−7		
Experiment 3				
Untreated	93			
Column passage	−5			

[a] Percent growth inhibition.

with the regular (C58NT)D but instead of returning to normal as the regressor tumor is rejected, the activity stayed elevated until the animal died with widespread metastases.

Characterization of the Cell Responsible for the GI-Activity

Experiments were performed to determine the nature of the cells responsible for the GI-activity. Experiment 1 shows that the GI-activity was completely abrogated by passage of the cells over a rayon column. Experiment 2 indicates the the activity was relatively radioresistant. Treatment with carrageenan resulted in incomplete abrogation of the activity, whereas iron and magnet treament completely eliminated the GI-activity. Treatment of cells with anti-rat immunoglobulin and complement had no deleterious effects on the GI-activity. In the normal spleen population, antithymocyte serum (ATS) and complement resulted in an increase in GI-activity. Similar experiments using spleens from animals bearing progressively growing tumors, gave the same pattern of results (Table 5). The growth inhibitory activity was abrogated by iron and magnet treatment as well as passage over a rayon column, and treatment with ATS and C enriched the activity. However, cells passed through a rayon column and then treated with ATS and C did not acquire activity.

Suppressor Cells in Normal Spleen Populations

The results in the GIA indicated that suppressor cells also might be present in the spleens of normal rats. To confirm this, normal spleen cells were directly tested for suppressor activity by addition as third party cells in the MLTI. The normal spleen cells were consistently found to inhibit the degree of stimulation by (C58-NT)D (Table 6). Selective removal of cells bearing surface immunoglobulin (Table 6) or of T cells, by treatment with ATS and complement (data not shown), did not abrogate that effect. Passage of the cells over a rayon column or treatment of

TABLE 6

Characteristics of Suppressor Cells in Normal Spleen

Stimulating cells	Responding cells		Responding cell + equal numbers of spleen cells treated with mmc as a source of suppressor cells	
	Reg-40	Reg-40 column passage	+ Normal spleen	+ Normal spleen column passage
Experiment 1				
Media	2,522[a]	1,475	2,463	588
(C58NT)D	24,269	46,676	14,616	31,524
	Reg-30	Reg-30 column passage	+ Normal spleen	+ Normal spleen anti-Ig and C
Experiment 2				
Media	4,659	7,785	4,242	2,958
(C58NT)D	6,763	29,827	10,517	8,515
	Reg-36	Reg-36 column passage	+ Normal spleen	+ Normal spleen iron and magnet
Experiment 3				
Media	10,738	15,488	7,840	16,072
(C58NT)D	54,639	128,860	36,421	88,859

[a] cpm [^{3}H]TdR.

the cells with carbonyl iron and magnet (Table 6) did abrogate the suppressor effect. This suggests that the cell found in normal spleens which can suppress immune lymphoproliferative responses to (C58NT)D in this system is also a macrophage.

DISCUSSION

The present investigation has extended the previous observations of suppressor cells in the spleens of rats bearing progressively growing (C58NT)D tumors (4) which can interfere with the lymphoproliferative response of immune cells in the MLTI. Suppressor cell activity was found in the spleens of normal rats and of rats after tumor regression. Suppressor cells which inhibit lymphoproliferative responses have been described in normal rat spleen cells before by Folch and Waksman (20). They have concluded that the suppressor cell is an adherent T cell primarily because few suppressor cells can be detected after adult thymectomy, irradiation, and bone marrow reconstitution. Our results would appear to be in

sharp contrast to their conclusions. However, the systems may not be comparable since they have used suppresion of mitogen responses and of primary proliferative responses to alloantigen to demonstrate the suppressor effect, and our data are limited to a secondary proliferative and cytotoxic response to a tumor associated antigen. We are currently studying the primary response of the normal rat spleen to alloantigens in an effort to sort out these differences.

In addition to suppressor activity, we have found rat spleens to have nonspecific cytostatic effects on several rapidly proliferating tumor cells *in vitro*. The data presented here indicate that, as in the mouse (5), similar or identical effector cells may be responsible both for the suppressor cell activity and for tumor growth inhibitory activity. The data supporting this concept are:

(1) The relative levels of suppressor and growth inhibitory activities under various circumstances were quite similar (e.g., Figs. 6 and 7).

(2) Suppressor cell activity in the spleens of rats (4) are particularly prominent in the presence of actively growing tumors. Similarly, GI-activity (Fig. 8 and Table 3) is increased in tumor-bearing rats.

(3) Significant levels of both activities have been detected in the spleens of non-tumor bearers. In contrast to our previous observations in mice (5), suppressor cell activity and GI-activity could be demonstrated in the spleens of normal rats as well as in rats after rejection of their tumors. Recent work in the mouse, indicating that GI-activity can be demonstrated in spleens of normal mice at very high E/T ratios (6), suggests the difference between mice and rats in terms of splenic GI-activity and probably suppressor activity is quantitative and not qualitative. That rats have more suppressor activity in the spleens of non-tumor bearers, as compared to mice, would help explain some of the difficulties frequently experienced using rat spleen cells as responders in assays of lymphoproliferative response. Of interest, a recent report of *in vitro* generation of cytotoxic lymphocytes in rat spleens mentions the requirement that adherent cells be removed from the spleen suspensions before the culture period in order to obtain cytotoxic lymphocytes (2). There is considerable evidence that the generation of such a primary cytotoxic response depends upon a proliferative event (28–30).

(4) In addition to the rather complete association of the GI-activity with suppressor activity, the effector cells have similar if not identical characteristics. The growth inhibitory cells were radioresistant, adherent, phagocytic, inactivated by carrageenan, lacked surface immunoglobulin, and were resistant to a specific anti-T cell serum. The suppressor cells in this system had the same characteristics and all of these data are compatible with a macrophage-mediated effect.

(5) The effector cells appeared to have a broad and nonspecific range of activity. Tumor cells without known common or cross-reacting antigens were similarly susceptible to the growth inhibitory effects. The lack of specificity of the cytostatic effects on rapidly proliferating tumor cells would be compatible with the suppressor activity of the same cells on the proliferative response in the MLTI of autologous or syngeneic lymphocytes. Our observations in this regard are consistent with the previous findings of Kirchner *et al.* in mice (5).

Some practical points can be drawn from these data. The ability to consistently detect lymphoproliferative responses to tumor associated antigens in the rat can be substantially enhanced by the removal of suppressor cells from the responding cell populations. Glaser *et al.* (4) previously showed that progressor spleen cells could

react in MLTI after removal of adherent or phagocytic cells. We have now shown the responses of regressor cells can be augmented by similar treatment. Our data already indicate that the kinetics of responsiveness in MLTI (i.e., the period of time after inoculation with tumor cells during which a positive MLTI can be demonstrated) is much broader than previously described. However, a careful kinetic study of the reactivity in MLTI to (C58NT)D of spleen cells, with suppressor cells removed, remains to be done. Even with the increased sensitivity of the assay, immunization with tumor antigens still appears to be required for reactivity in the MLTI, since normal spleen cells did not react.

Since the suppressor and GI-activities were well correlated, it appears that the GIA can be used as a convenient substitute for the suppressor cell assay. Screening for suppressor cell activity by the GIA would have several advantages. The GIA is a more rapid, simple assay, permitting larger tests to be performed. Furthermore, the GIA is not dependent on the reactivity of the responding immune cells or the presence of sufficient antigen for stimulation which can present problems in a suppressor cell assay using the MLTI. Also, only two cell types, instead of three, are needed in the tests, and the effector cells do not have to be blocked or otherwise treated to avoid lymphoproliferative responses.

Of central importance is the *in vivo* role of these cytostatic macrophages. On the one hand, these cells would seem to have considerable immunodepressive effects. Not only was the proliferative response in MLTI suppressed but also the *in vitro* generation of a secondary cytotoxic response was inhibited. On the other hand, however, these macrophages have the ability to inhibit the proliferation of tumor cells *in vitro* and might also have such effects *in vivo*. Studies are presently in progress to determine whether the immunosuppressive or anti-tumor effects play a predominant role *in vivo* under various conditions of tumor growth.

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