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# Immune Response to Polyoma Tumor Cells in Mice—III. Stimulation of Tumor Cell Growth *in Vitro* by Spleen Cells from Immunized Animals*

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**Abstract**—*We have evaluated the stimulation of target cell growth* in vitro *by spleen cells from mice which were immunized with polyoma-transformed cells and other tumor and non-tumor antigens. Stimulation was particularly seen under conditions of immunization that were suboptimal for the production of specific cytotoxicity. Significant stimulation of polyoma target cell growth was observed by lymphocytes from mice immunized against* $10^5$ *Py 4198 tumor cells. This stimulation of target cell growth was not confined to polyoma-transformed cells only. Cells transformed by SV40, H-MuSV and non-transformed cells like 3T3 and embryo fibroblasts were also stimulated. Immunization of mice with syngeneic embryo fibroblasts also resulted in stimulation of tumor cell growth by the spleen cells from the immunized mice. However, the growth stimulation was less consistent and did not occur in all target cells tested. The specificity of immunostimulation was further studied with the Moloney sarcoma virus (M-MuSV) system; an antigenically distinct tumor system. Spleen cells from M-MuSV tumor-bearing mice stimulated cell growth* in vitro *not only against MuSV-transformed cells but also with SV40-transformed and polyoma-transformed cells as targets. Significant stimulation of target cell growth was also observed by spleen cells from mice that were immunized against 'non-pertinent' antigens, e.g. sheep red blood cells and allogeneic (C57B1/6) spleen cells.*

## INTRODUCTION

POLYOMA virus has been investigated as a model of a cancer-producing DNA virus. Polyoma virus inoculated subcutaneously into mice induces tumors after a latent period of several months. The natural host for polyoma virus is the mouse, and under ordinary circumstances immunocompetent hosts do not develop polyoma tumors. However, neonatal infection of susceptible hosts will result in the development of polyoma tumors [1, 2]. Some animals are resistant to the development of polyoma tumors even upon neonatal infection [3]. However, immunosuppressive procedures such as neonatal thymectomy will result in the development of tumors, even in strains which are resistant. Cells transformed by oncogenic DNA viruses possess non-virion antigens not found in or on normal adult tissues [4–11]. Some of these antigens can provoke an immune response resulting in rejection of syngeneic tumor cells following appropriate pre-immunization.

We have found that $10^6$ polyoma-transformed cells used for the last injection of a hyperimmunization schedule in syngeneic mice produced maximum specific lymphocyte cytotoxicity. At this dose, the cytotoxicity was specific for cell lines transformed by polyoma virus and no significant cytotoxicity was observed against cell lines transformed by other viruses. Immunization with $10^7$ polyoma-transformed cells, however, resulted in loss of specific cytotoxicity for the cell lines transformed by polyoma virus, but produced a strong cytotoxicity against simian virus 40 (SV40)-transformed cells. We also found that immunization against primary sygeneic embryo fibroblasts induced cell-mediated cytotoxicity against polyoma tumor cells. However, the cytotoxicity was generally weaker than that

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observed by spleen cells from mice immunized with the same regimen of polyoma-transformed cells and was observed against all tumor cell lines tested. In addition, spleen cells from animals immunized against embryo fibroblasts often caused stimulation of tumor cell growth *in vitro* instead of cytotoxicity [12]. Antisera from these animals were found to induce cell-mediated cytotoxicity by normal spleen, thymus and lymph node effector cells [13]. The antibody-dependent cell-mediated cytotoxicity (ADCC) was predominantly specific for polyoma-transformed cells when the sera came from polyoma tumor-immunized mice. In contrast, antisera from embryo fibroblast-immune mice induced ADCC against all tumor cells tested, including polyoma, SV40 and MuSV-transformed cells [13].

There have been a number of reports indicating stimulation of tumor growth by a weak immune response whereas a strong response prevents the growth. Immunostimulation has been observed with chemically and virally induced tumors. Prehn's experiments suggested that chemically induced tumor cells, inoculated into thymectomized, irradiated, syngeneic recipients, grew slightly better if administered with immune spleen cells than with normal spleen cells when both were given at low concentrations[14, 15]. The *in vitro* correlate for immunostimulation was proposed to be the specific stimulation of tumor cell growth by small numbers of tumor-immune lymphocytes [15].

In *in vitro* studies of cell-mediated immunostimulation of tumor growth, Fidler suggested that Con-A-stimulated rat lymphocytes significantly enhanced the growth of the B16 melanoma whereas normal lymphocytes had little or no effect [16]. Cell-mediated immunostimulation induced by mammary tumor virus-free BALB/c mammary tumors was tested by Medina and Heppner[17]. They detected cell-mediated inhibition with a lymphocyte:target ratio of 1000:1 and stimulation was detected at ratios of 100:1 or 10:1. Murasko and Prehn [18] have also shown the stimulatory effect of immunization on tumor induction by Moloney murine sarcoma virus. They suggested that immunization with virus can either stimulate or inhibit virus-induced tumorigenesis, depending on the dose of virus used for immunization. Their interpretation indicated that low amounts of immune cells or sera stimulated tumor cell growth whereas large amounts inhibited tumor cell growth [18].

Our studies on the immune response to polyoma tumor cells have shown the impact of graded numbers of polyoma tumor cells used as the last immunizing dose on lymphocyte cytotoxicity [12]. Whereas $10^6$ polyoma-transformed cells for the last injection of a hyperimmunization schedule appears to be an optimum dose for the production of a specific lymphocyte cytototic response, $10^5$ cells produced no cytotoxic lymphocytes but enhanced tumor cell growth *in vitro*.

The purpose of the present investigation was to further study the stimulation of tumor cell growth *in vitro* by immune lymphocytes and to evaluate the specificity of the reaction in the Polyoma System.

## MATERIALS AND METHODS

### *Animals*

Adult C3H mice of both sexes were used as a source of immune and control lymphocytes. Control animals were matched with experimental animals by sex and age.

### *Virus*

M-MuSV, Moloney strain of murine sarcoma virus lot MSV-B-122, prepared from mouse tumors (BALB/c), was provided by the Office of Progress Resources and Logistics, Viral Oncology, National Cancer Institute, Bethesda, MD.

### *Cell lines*

Py 4198 is a polyoma-transformed cell line of C3H origin, SVA 31 Cl-4 (SVA) and SVT-2 are SV40-transformed cell lines of BALB/c origin. These cell lines were kindly provided by Dr. Ron Herberman, National Cancer Institute, Bethesda, MD. Py3T3, a polyoma virus-transformed cell line of NIH Swiss origin, was obtained from Dr. G. Todaro, NIH, Bethesda, MD. Ha2 is an *in vitro* cell culture line derived from a murine sarcoma virus-induced tumor of a CBA ($H\text{-}2^k$) mouse [19]. Primary C3H embryo fibroblasts were obtained from 7 to 15-day-old embryos. SEYF is a polyoma-transformed cell line of ABY ($H\text{-}2^b$) origin and was kindly provided by Dr. G. Klein, Stockholm, Sweden.

### *Immunizations*

(a) At weekly intervals, C3H mice were inoculated i.p. with $1 \times 10^5$ Py 4198 cells treated with 25 $\mu$g mitomycin C/ml at 30°C for 30 min. The experiments were performed 4 days after the last injection.

(b) Another group of animals were similarly immunized with primary C3H embryo fibroblasts. Primary embryo explants were obtained

from primiparous mice and the cultured embryo fibroblasts were used within 1 week.

(c) A third group of C3H animals were injected i.p. with 0.1 ml of 0.25% sheep red blood cells (SRBC). The spleen cells were tested 5 days after immunization.

(d) A separate group of C3H animals were injected with $1 \times 10^7$ mitomycin C-treated spleen cells from C57B1/6 mice. Spleens were harvested from these animals 5 days after immunization.

(e) C3H animals also received 0.1 ml of M-MuSV suspension i.m. in the thigh, after which local tumors developed as early as 5 days, reaching a peak tumor size of 10–15 mm at about day 15, when spleen cells were harvested from these animals.

*Medium*

Eagle's MEM with 10% heat-inactivated FCS and 100 $\mu$g gentamicin/ml was used for all incubations and dilutions.

*Lymphoid cells*

Spleens and thymuses were dissected out under aseptic conditions and prepared separately. Thymuses were dissected out following wide exposure of the mediastinal and thoracic cavities, carefully visualizing each lobe of the thymus to avoid possible contamination with parathymic nodes. The organs were pressed through fine nylon mesh into cold MEM without FCS, mixed with a Pasteur pipette and passed over a fine nylon mesh filter. The cells were pelleted by centrifugation, resuspended in Tris(hydroxymethyl)-aminomethane-buffered 0.75% ammonium chloride (pH 7.2) and incubated for 10 min at 37°C to lyse the erythrocytes. The cells were centrifuged, resuspended in 5 ml of complete MEM, washed three times and resuspended in complete MEM. Appropriate dilutions of immune spleen, control spleen or thymus were then used in microcytotoxicity assays.

*Microcytotoxicity tests*

A modification of the microcytotoxicity assay of Takasugi and Klein [20] was used. Microplates (3034, Falcon plastics, Division of BioQuest) were seeded with 200 target cells per well in volumes of 5 $\mu$l and incubated for 20 hr at 37°C in 5% $CO_2$. Immune and normal lymphoid cells were then added in volumes of 5 $\mu$l per well. The highest concentration of lymphoid cells used was 20,000/well and the lowest was 2500/well. Parallel concentrations of immune and control lymphoid cells were always tested on the same plate. After incubation for 20 hr the medium was removed, the plates were fixed and stained, and the remaining target cells in each well were counted.

*Evaluation of results*

The logarithmic mean and standard deviation of the target cells in the six replicate wells of each lymphocyte dilution were determined and Student's *t*-test was performed to assess the significance of the differences between numbers of target cells in wells containing immune lymphocytes and those containing the same number of control lymphocytes. A significant difference was considered to exist when the *P*-value was less than 0.05. The relative percentage of remaining target cells in wells containing immune lymphocytes was calculated compared to wells containing the same number of control lymphocytes. The percentage of surviving target cells is based on the following formula:

$$\frac{\text{Number of target cells in wells with immune lymphocytes}}{\text{Number of target cells in wells with control lymphocytes}} \times 100.$$

The results of microcytotoxicity assays reflect a variety of factors that can influence the number of cells remaining in the wells at the end of the incubation time, as discussed in detail elsewhere [21]. These include cell growth, cytotoxicity, detachment of target cells without cytotoxicity and inhibition of cell growth by depletion of nutrients and accumulation of waste products. We have used logarithmic calculations in evaluating our data because the results are clearly influenced by cell growth. We have chosen to evaluate the significance between groups within a given experiment using Student's *t*-test rather than relying on the mean percentage survival of target cells as an indicator of differences. This reliance on statistical analysis was done for two reasons: firstly, in experimental groups having a relatively high variability among samples, the mean value may be much higher than others within the same experiment having a lower variability in surviving cell numbers. Thus, in the former cases, larger mean numbers may not be significantly different from the control values whereas the latter cases with lower variability may be significantly different from the control; secondly, because the microplate assay encompasses multiple variables, as indicated above, clear titration effects are not always

seen. That is, clearly significant negative or positive slopes are not always obtained in these experiments. Previous investigators have used effector:target cell ratios as high as 1000:1, where inhibition of target cell growth was found; growth stimulation was detected at ratios of 100:1 to 10:1 [17]. The highest ratio that we have tested has been an effector:target cell ratio of 100:1 (20,000 lymphoid cells per 10 $\mu$l, or $2 \times 10^6$/ml). This concentration of cells is clearly at the upper limit of cells that can be supported by the tissue culture medium for a 20-hr period.

In addition to evaluating pairs of data by Student's *t*-test, we also analyzed the slopes using a linear regression analysis of the mean percentage of surviving target cells vs the effector:target cell ratio with a statistical analysis system (SAS) developed by the SAS Institute at Raleigh in North Carlina. These analyses were done using an IBM 370, Model 158 computer at the University of Alabama in Birmingham.

*Specificity controls*

To evaluate the specificity of immunostimulation, we have used the following specificity controls which have been used previously in tumor cell cytotoxic reactions [12, 21].

(1) The activity of immune lymphocytes was compared to the activity of control lymphocytes from non-immune animals. ('Immune' spleens were also compared, in selected experiments, to normal thymus to exclude the possibility that differences were due to decreases in natural killer (NK) activity.)

(2) The activity of immune lymphocytes was compared to the activity of control lymphocytes that were immune to a 'non-pertinent' (i.e. non-tumor) antigen.

(3) The immune lymphocytes were tested on control target cells which were either transformed by other tumor viruses or were untransformed.

(4) Reciprocal specificity controls were performed with an antigenically distinct tumor.

## RESULTS

In evaluating immune responses to polyoma tumors in mice, we performed a series of experiments using different doses of polyoma-transformed cells. Specific cytotoxicity was found only when the mice were hyperimmunized with $1 \times 10^6$ polyoma-transformed cells as the last injection [12]. When the final immunizing dose of polyoma-transformed cells was suboptimal, stimulation of target cell growth was observed.

Table 1 shows the stimulation of polyoma-transformed target cell growth by spleen cells obtained from mice immunized once, twice and four times with $10^5$ Py 4198 tumor cells. Significant stimulation of the target cells is indicated by an asterisk. Three different polyoma-transformed cell lines from different mouse strains were used and significant stimulation of cell growth was observed in all three. Significant stimulation of polyoma-transformed target cells was produced in each experiment by at least one dilution of effector cells. In the four experiments, 11 out of 13 microplates showed significant growth stimulation by the 'immune' spleen cells compared to the growth of the target cells in wells with control lymphocytes from uninjected animals.

To investigate if this stimulation of target cell growth was confined to polyoma-transformed cells, we tested cell lines transformed by other viruses as well as untransformed cells. Table 2 shows the stimulation of growth of these 'control' target cells by spleen cells from mice immunized against $10^5$ Py 4198 tumor cells. All the cell lines tested showed significant stimulation of growth by at least one of the E:T ratios used (16 out of 16 microplates). The spleen cells used to generate the data shown in Tables 1 and 2 came from the same animals. Therefore it is apparent that this stimulation of target cell growth by spleen cells obtained from mice immunized against Py 4198 tumor cells is not specific for polyoma-transformed cell lines, as other cell lines that were non-transformed or transformed by SV40 or H-MuSV were also stimulated to grow.

It was conceivable that the observed stimulation could be due to some common (for example, embryonic) antigen. Thus we tested immune spleen cells obtained from mice immunized against embryo fibroblasts. Significant stimulation of target cell growth was also observed by these spleen cells from embryo fibroblast-immunized animals (Table 3). The incidence of this stimulation of cell growth by embryo fibroblast-immune cells in terms of the various target cells tested, however, was lower than that observed with the polyoma-'immune' spleen cells; i.e. 5 out of 12 microplates tested showed significant stimulation. Interestingly, the embryo fibroblasts and Py 4198 cells were not stimulated significantly in these experiments.

As a further specificity control we tested several of the target cells against spleen cells from mice injected with Moloney sarcoma

*Table 1. Stimulation of polyoma tumor cell growth by spleen cells obtained from mice immunized against Py 4198 tumor cells*

| Experiment No. | Immunization schedule | Target cells | % Control 50:1 | 40:1 | 30:1 | 20:1 | 10:1 |
|---|---|---|---|---|---|---|---|
| | 1° | | | | | | |
| 25 | | Py 3T3 *a* | †60 | 68 | 136 | 193* | 177 |
| | | Py 4198 *a* | †50 | 79 | 176* | 179* | 196 |
| | 2° | | | | | | |
| 26 | | Py 3T3 *a* | 92 | 95 | 187* | 114 | 126 |
| | | Py 4198 *a* | †64 | 84 | 155 | 133 | 170 |
| | 4° | | | | | | |
| 38 | | Py 4198 *a* | 159* | 165* | 115 | 130* | N.D. |
| | | Py 4198 *b* | †76 | 95 | 127 | 147* | N.D. |
| | | Py 4198 *c* | 155* | 113 | 164* | 193* | N.D. |
| | | | 100:1 | 75:1 | 50:1 | 25:1 | |
| | 4° | | | | | | |
| 44 | | Py 4198 *a* | 107 | 107 | 114 | 150* | |
| | | SEYF *a* | 118 | 148* | 129* | 144* | |
| | | Py 4198 *b* | 110 | 108 | 113 | 147* | |
| | | Py 3T3 *a* | 149 | 71 | 104 | 108 | |
| | | SEYF *b* | 119 | 78 | 157* | 140* | |
| | | Py 4198 *c* | 88 | 146 | 135 | 236* | |

% Control calculated as shown in the text.
Immunization schedule: 1° indicates primary injection of $1 \times 10^5$ polyoma transformed cells; 2° means two injections of $1 \times 10^5$; 4° means four injections of $1 \times 10^5$.
Target cells: *a*, *b* and *c* represent different animal spleens tested on the same target cell line.
N.D. = Not done.
*Significant stimulation, $P < 0.05$.
†Slope (% control vs E:T ratio) significantly negative.

*Table 2. Spleen cells from mice immunized against Py 4198 tumor cells stimulate growth of target cells not transformed by polyoma virus*

| Experiment No. | Immunization schedule | Target cells | % Control E:T ratio 50:1 | 40:1 | 30:1 | 20:1 | 10:1 |
|---|---|---|---|---|---|---|---|
| | 1° | | | | | | |
| 25 | | 3T3 *a* | 123 | 238* | 76 | 71 | 116 |
| | 2° | | | | | | |
| 26 | | SVT *a* | 23 | 69 | 105 | 165* | 92 |
| | | Embryo fibroblast *a* | †51 | 49 | 71 | 98 | 221* |
| | | 3T3 *a* | †81 | 100 | 104 | 115 | 160* |
| | | 3T3 *b* | 150 | 84 | 173* | 234* | 130 |
| | 4° | | | | | | |
| 38 | | Embryo fibroblast *a* | 109 | 144* | 115 | 119* | |
| | | SVT *a* | 132 | 147* | 146* | 86 | |
| | | SVT *b* | 141 | 116 | 170* | 146* | |
| | | | 100:1 | 75:1 | 50:1 | 25:1 | |
| | 4° | | | | | | |
| 44 | | SVT *a* | 201* | 96 | 139 | 137 | |
| | | SVA *a* | 143* | 147* | 94 | 112 | |
| | | Ha2 *a* | 143* | 103 | 109 | 150* | |
| | | Embryo fibroblast *a* | 66 | 85 | 119 | 220* | |
| | | SVT *b* | 228* | 82 | 88 | 139* | |
| | | SVA *b* | 171 | 121 | 120 | 139* | |
| | | Ha2 *b* | 180 | 124 | 130* | 127* | |
| | | Embryo fibroblast *b* | 236* | 70 | 107 | 123 | |

Immunization schedule: as shown in Table 1.
% Control calculated as shown in the text.
*Significant stimulation, $P \leq 0.05$.
Target cells *a* and *b* represent duplicate assay with the same target cell line using different animals. *a* and *b* in this table correspond to *a* and *b* in Table 1 also.
†Significant negative slope.

virus as representative of a distinct or 'irrelevant' tumor. We have previously shown that MuSV-transformed cells are stimulated to grow *in vitro* by spleen cells obtained from mice injected with M-MuSV 15 days prior to the assay [22]. We thus chose this time after injection to further evaluate the specificity of this stimulation.

Table 4 shows the stimulation of target cell growth by spleen cells obtained from mice injected with Moloney sarcoma virus. Polyoma, H-MuSV and SV40-transformed cell lines all showed significant stimulation of growth by these spleen cells. Indeed, the greatest magnitude of *in vitro* cell growth stimulation was observed in these experiments.

The data thus far were consistent with immunostimulation specific for some common antigen(s) shared by all the target cells tested. To further test the specificity of this stimulation, we immunized mice against 'non-pertinent' antigens, i.e. sheep red blood cells and allogeneic (C57B1/6) splenocytes. The results are summarized in Tables 5 and 6. All the cell lines tested showed significant stimulation of target cell growth by at least one of the E:T ratios. It was conceivable that the 'immunostimulation' of cell growth *in vitro* we have observed in these experiments was rather an inhibition of natural killer cell activity since we have compared our results to normal spleen cells. However, as shown in Table 6 when the

*Table 3. Stimulation of target cell growth by spleen cells obtained from mice immunized against C3H embryo fibroblasts*

| Experiment No. | Target cells | % Control E:T ratio 100:1 | 75:1 | 50:1 | 25:1 | 12.5:1 |
|---|---|---|---|---|---|---|
| 41 | Py 3T3 *a* | †139* | 130 | 126 | 121 | 120 |
| | Py 3T3 *a* | 116 | 140* | 119 | 129* | 172* |
| | Py 4198 *a* | ‡61 | 77 | 73 | 99 | 105 |
| | Py 4198 *a* | 82 | 80 | 87 | 98 | 91 |
| | SEYF *a* | 114 | 95 | 87 | 91 | 93 |
| | Ha2 *a* | 99 | 84 | 116 | 98 | 132 |
| | SVT *a* | 152* | 182* | 216* | 142* | 138* |
| | Embryo fibroblast *a* | 105 | 108 | 103 | 116 | 72 |
| | Embryo fibroblast *b* | 96 | 115 | 98 | 110 | 109 |
| | Ha2 *b* | 116* | 118* | 119* | 106 | 82 |
| | SVA *b* | 76 | 111 | 111 | 80 | 106 |
| | SEYF *b* | 87 | 92 | 106 | 121* | 94 |

Immunization schedule: four weekly injections of $10^5$ embryo fibroblasts.
Target cells: *a* and *b* represent different spleen cell donors.
Py 3T3 and 4198 were tested in duplicate assays using spleen cells from animal *a*.
% Control as explained in Tables 1 and 2.
*Significant stimulation, $P \leq 0.05$.
†Significant positive slope.
‡Significant negative slope.

*Table 4. Stimulation of target cell growth by spleen cells obtained from mice injected with Moloney sarcoma virus*

| Experiment No. | Animal No. | Target cells | % Control E:T ratio 100:1 | 50:1 | 25:1 | 12.5:1 |
|---|---|---|---|---|---|---|
| 105 | 1 | Py 4198 | †1128* | 724* | 677* | 580* |
| | 2 | Py 4198 | †522* | 388* | 354* | 259* |
| | 1 | Ha2 | 147* | 116 | 90 | 119 |
| | 2 | Ha2 | †136* | 94 | 81 | 78 |
| | 1 | SVT | 270* | 269* | 198* | 129 |
| | 2 | SVT | 180* | 581 | 168* | 137 |

% Control as explained in the text.
*Significant stimulation, $P \leq 0.05$.
†Significant positive slope.

*Table 5. Stimulation of target cell growth by spleen cells obtained from C3H mice immunized against xenogeneic erythrocytes*

| Target cells | % Control E:T ratio 100:1 | 75:1 | 50:1 | 25:1 | 12.5:1 |
|---|---|---|---|---|---|
| SVT | N.D. | 148* | 190* | 178* | N.D. |
| D56 | N.D. | 120 | 235* | 507* | 148* |
| Ha2 | 82 | 123* | 89 | 107 | 80 |

N.D. = Not done.
% Control calculated as shown in the text.
*Significant stimulation $P \leq 0.05$.

stimulation of target cell growth by alloimmune spleen cells was compared with normal spleen and normal thymus, the magnitude of stimulation was also singificant when compared to normal thymus. Since normal unfractionated thymus cells are devoid of NK activity, these data strongly suggest that the observed differences in target cell number at the end of the assay was due to increased cell growth in the wells containing immune spleen cells.

## DISCUSSION

We have conducted this series of experiments in an attempt to evaluate the specificity of stimulation of tumor target cell growth *in vitro* by lymphocytes from mice injected with polyoma-transformed tumor cells (Py 4198). Stimulation was seen particularly under conditions of immunization that were suboptimal for the production of cytotoxicity. Spleen cells from mice immunized against polyoma-transformed cells produced significant stimulation of cell growth not only of polyoma-transformed cell lines but also of SV40 and H-MuSV-transformed cells, as well as untransformed cells like 3T3 and embryo fibroblasts. The stimulation did not appear, therefore, to be specific for polyoma-transformed cell lines, nor was it restricted by the strain of origin of the target cells. The specificity of cell-mediated cytotoxicity in this system previously reported [12] was dependent on the immunization schedule. At the optimum dose, cytotoxicity was specific for cell lines transformed by polyoma, and no cytotoxicity was observed against cell lines transformed by other viruses [12]. The data from polyoma tumor cell-immunized mice shown in the present report are from animals receiving $10^5$ cells 1–4 times. This was suboptimal for induction of cytotoxicity [12]. Animals immunized with $10^6$ cells generated optimum specific cytotoxic effects whereas spleen cells from animals immunized with $10^7$ cells at the last injection of an hyperimmunization schedule produced two different effects: (a) non-specific cytotoxicity; and (b) inconsistent stimulation of target cell growth of polyoma-transformed cells [12].

Since the stimulation of target cell growth was not specific for polyoma-transformed cells, we thought that different target cell lines might be expressing common antigen(s), for example embryonic antigen(s), and that weak immunologic reactivity against such antigens is responsible for stimulating target cell growth (see Table 3). This supposition, however, would not explain the increase in target cell growth produced by immunization against 'non-per-

*Table 6. Stimulation of target cell growth by spleen cells obtained from C3H mice immunized against allogeneic spleen cells. Comparison of stimulation of immune spleen vs normal thymocytes and spleen cells*

| Experiment No. | Immune to: | Target cells | Lymphoid cells† | % Control E:T ratio 100:1 | 50:1 | 25:1 | 12.5:1 |
|---|---|---|---|---|---|---|---|
| 101 | C57B1/6 Spleen | SVT *a* | Imm SP/NSP | 195* | 160* | 135 | 55 |
| | | SVT *a* | Imm SP/NTx | 83 | 252* | 439* | 161* |
| | | Py 4198 *a* | Imm SP/NSP | 130 | 215* | 167 | 132 |
| | | Py 4198 *a* | Imm SP/NTx | 110 | 133 | 174* | 114 |
| | | SVT *b* | Imm SP/NSP | 200* | 206* | 244* | 247* |
| | | SVT *b* | Imm SP/NTx | 477* | 418* | 472* | 289* |
| | | Py 4198 *b* | Imm SP/NSP | 110 | 163* | 198* | 149 |
| | | Py 4198 *b* | Imm SP/NTx | 283* | 274* | 228* | 98 |

Spleen cells obtained from immunized mice were compared to normal spleen cells or with normal thymocytes for stimulation of target cell growth using the formula in the Materials and Methods section.
*a* and *b* indicate different animals used as sources of immune spleen cells.
*Significant stimulation, $P \leq 0.05$.
†Lymphoid cells: Imm SP = immune spleen cells; NSP = normal spleen cells; NTx = normal thymus cells.

tinent' antigens like SRBC or allogeneic spleen cells (Tables 5 and 6). To further test the specificity of immunostimulation, we performed reciprocal specificity controls with an antigenically distinct tumor, namely M-MuSV. Stimulation was produced not only against MuSV-transformed cells as previously demonstrated [22], but also with SV40-transformed cells and polyoma-transformed cells as targets.

We also attempted to analyze whether low concentrations of effector cells produced more significant stimulation of target cell growth than high concentrations of effector cells, as has been reported previously [17]. To do this we asked if the slope within a given experiment (mean percentage target cell survival vs effector:target cell ratio) was significantly positive or negative. Analysis of the data in Table 1 revealed that four of the individual microplates tested showed a significantly negative slope. Also, when all of the data in the table were analyzed by regression analysis, the slope was found to be negative and significant. Thus for this set of experiments, the trend of increased stimulation of tumor target cell growth by smaller concentrations of immune lymphoid cells appears to be statistically correct. However, this trend was not supported by analyses of the data presented in Tables 2–6. Also, as one can see in all of the tables, the results from individual microplates often did not reveal clear titration effects. In Table 2, data from only 3 of the 16 microplates tested revealed significantly negative slopes. In Table 3, data from one microplate represented a significant positive slope, and from one microplate a significant negative slope was obtained. Data from 3 out of 6 microplates represented in Table 4 have significant slopes in a positive direction; that is, increasing growth stimulation was correlated with increasing effector cell numbers. In Tables 5 and 6 no clear titration effects could be discerned by regression analysis, either positively or negatively.

It is conceivable that polyclonal activation of cells by immunization with 'non-pertinent' antigens could activate specific immunostimulation vs some shared antigen such as embryonic antigen. Also, it is conceivable that specific and non-specific immunostimulation co-exist and we have not been able to distinguish these. Inhibition of immunostimulation by purified soluble antigens or adsorption studies might be able to differentiate these two (specific vs non-specific stimulation). It would seem from the present experiments that growth stimulation of murine fibroblastoid cells *in vitro* is a property of activated lymphoid cells. The subpopulation(s) of cells responsible for this increased target cell growth has not been determined, nor has whether cell–cell contact is required. It is possible that the activated cells produce a lymphokine that stimulates DNA synthesis in homologous cells [23].

One must also consider the possibility that the observed 'stimulation' of tumor cell growth was an inhibition of NK activity, i.e. producing a false 'cell growth' in the experimental groups compared to the natural cytotoxicity of the control spleen. This does not seem likely, however, since stimulation in selected experiments was also significant in comparison to cells incubated with normal unfractionated thymocytes which are devoid of NK activity.

The *in vivo* relevance of the current findings with respect to tumor cell growth enhancement *in vivo* is not known. However, the specificity of the reactions in the classical immunologic sense may not be relevant to the *in vivo* significance. It is conceivable that non-specific growth stimulation could be a major antagonist to NK activity [24], which is also not antigen-specific in the classical immunologic sense.

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