Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 2209-2213, April 1980
Immunology

Suppression of tumor rejection by autologous anti-idiotypic immunity

(T lymphocytes/immune regulation/immunological unresponsiveness)

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Communicated by Hewson H. Swift, December 26, 1979

ABSTRACT Autologous anti-idiotypic responses to tumor-specific lymphocytes altered the capability of mice to reject syngeneic tumors. This was shown by using two non-cross-reacting fibrosarcoma lines, 1591 and 1316, induced by ultraviolet light. Cells from these tumor lines are regularly rejected when transplanted into normal syngeneic C3H mice but grow progressively in animals immunosuppressed by irradiation with ultraviolet light or by x-irradiation and thymectomy. Immunization of normal mice with 1591-specific lymphoblasts that had been generated in mixed lymphocyte-tumor cell cultures caused a loss of resistance to 1591 tumor cells, but the animals remained resistant to 1316 tumor cells. *In vitro,* spleen cells from animals immunized with 1591-specific lymphoblasts did not generate cytolytic T cells to 1591 fibrosarcoma cells, but spleen cells from the same animals responded normally to 1316 fibrosarcoma cells. Furthermore, spleen cells from animals immunized with 1591-specific lymphoblasts contained idiotype-specific T cells that lysed 1591-specific lymphoblasts, whereas 1316-specific lymphoblasts were unaffected. Immunization of normal animals with nonresponding lymphocytes from the same mixed lymphocyte-tumor cell cultures as the 1591-specific lymphoblasts showed normal responses to both tumors *in vivo* and *in vitro.* These results suggest that changes in the balance of tumor-specific and anti-idiotypic T lymphocyte clones can influence the capability of an individual to respond effectively to tumor antigens and can determine whether a tumor grows or regresses.

The influence of the immune system on tumor development and growth has been intensively investigated for several decades. It is apparent that many different immunologic reactants—such as macrophages, naturally occurring killer cells, and tumor-specific lymphocytes—can destroy cancer cells. Furthermore, immune cells may also be part of a host protective mechanism that appears to be operating during tumorigenesis (1). However, numerous clinical and experimental immunotherapeutic trials have shown that our understanding of the complex regulation of tumor-specific immune responses is not sufficient to devise regularly effective immunotherapeutic approaches.

The present study examines the question of whether anti-idiotypic immunity can regulate tumor-specific immune responses and control tumor growth and regression. An anti-idiotypic immune response is directed against antigenic determinants on an individual's own immunoglobulin or T cell receptors. In selected nontumor systems, anti-idiotypic immunity has been shown to regulate effectively and specifically the immune responses of the lymphocyte clones expressing the idiotype (2-12). However, little is known about possible influences of such immunity on the regulation of tumor-specific immunity and tumor growth. Clinical studies have shown that anti-idiotypic immunity can develop in human cancer patients and that the development of such immunity appears to correlate with metastatic progression and disappearance of tumor-specific immunity (13, 14). We demonstrate here that animals can develop an anti-idiotypic autoimmune reaction to tumor-specific T lymphocytes, and that such animals permit progressive growth of malignant cells expressing the tumor antigen.

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MATERIALS AND METHODS

Mice. Five- to ten-week-old female C3H/HeN (mammary tumor virus-negative) mice from a colony of germfree-derived, specific pathogen-free animals at the National Cancer Institute Frederick Cancer Research Center were used for the experiments.

Fibrosarcoma Lines. The two fibrosarcomas 1591 and 1316 were recently induced in C3H/HeN (mammary tumor virus-negative) mice by repeated exposure to UV light (15). The *in vitro* lines of the 1591 and 1316 fibrosarcomas used in our experiments were adapted to culture from the first transplant generations. Both the *in vivo* and *in vitro* lines of these tumors are strongly immunogenic and, when transplanted into syngeneic mice as tumor cell suspensions or tumor fragments, regularly regress after an initial growth during the first 10 days. In mice that were immunosuppressed by either UV-irradiation or thymectomy and x-irradiation (15, 16), tumors regularly grow and kill the animals by infiltrative growth and direct extension of the tumor into vital organs without macroscopic evidence of distant metastases. We have observed the development of a progressively growing variant tumor in only 1 of more than 300 normal animals injected with 1591 tumor cells. Spleen cells from animals injected with the variant tumor cells do not generate cytolytic T cells specific for the parental 1591 tumor cells when restimulated in culture. All fibrosarcoma lines were grown in minimal essential medium (GIBCO) containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

Mixed Lymphocyte-Tumor Cell Cultures (MLTC). Culture medium was Dulbecco's modified Eagle's medium (GIBCO H-21) supplemented with 5% heat-inactivated fetal calf serum, 470 μg of L-glutamine per ml, 580 μg of L-arginine per ml, 180 μg of L-asparagine per ml, 60 μg of folic acid per ml, 550 μg of sodium pyruvate per ml, 1% penicillin/streptomycin (GIBCO no. 600-5140), and 50 μM 2-mercaptoethanol (Calbiochem) added immediately before use. Spleens were aseptically removed, pressed through a wire screen, and further dispersed with a pasteur pipette. The cells were then washed three times, the erythrocytes were lysed by treatment with

Abbreviations: MLTC, mixed lymphocyte-tumor cell culture; NRL, nonresponding lymphocytes.

0.83% (wt/vol) ammonium chloride, and the cells were washed again in culture medium. MLTCs were made by adding 7×10^6 responder lymphocytes and 2×10^4 mitomycin C-treated stimulator cells in 2.0 ml of medium per culture (24-well tissue culture plate, Costar 3524).

Generation and Purification of Tumor-Specific Lymphoblasts. Mice were immunized with viable 1-mm^3 fragments of the 1591 tumor implanted subcutaneously with a trocar into both inguinal regions. Spleen cells from tumor-immune animals were removed sterilely 30 days after the primary immunization. Lymphocytes were restimulated in an MLTC by adding 7.5×10^5 mitomycin C-treated 1591 tumor cells to 2.6×10^8 lymphocytes in a 75-cm^2 tissue culture flask (Costar 3705) containing 75 ml of medium. After 5 days in culture, tumor-specific lymphoblasts were separated from nonresponding lymphocytes (NRL) by equilibrium density centrifugation (17). Briefly, cells harvested from 5-day cultures were washed three times with medium and resuspended in a solution of bovine serum albumin ($\rho = 1.082$ g/ml) that was overlayed with a less dense solution of bovine serum albumin ($\rho = 1.060$ g/ml). Suspensions were spun to equilibrium at $10{,}000 \times g$ for 20 min. The small dense NRL formed a pellet at the bottom of the tube, while the less dense lymphoblasts floated at the interface of the two solutions. The purity of the separated fractions was routinely determined by three different criteria: size, specific cytolytic activity, and [^{3}H]thymidine uptake. When compared to the high-density lymphocytes in the pellet, the low-density floating fraction consisted of more than 95% of the large lymphocytes and contained more than 90% of the cells incorporating [^{3}H]thymidine. The low-density lymphocytes also contained all of the cytolytic activity; they regularly showed 50–60% specific lysis to 1591 tumor cells but did not react with 1316 tumor cells (0–3% specific lysis).

Immunization of Animals with Purified Lymphocytes. The induction of anti-idiotypic immunity by immunization with syngeneic alloantigen-specific T lymphoblasts has recently been described (7–10, 18). We have adapted this protocol for the induction of anti-idiotypic immunity to tumor-specific antigens. A suspension of purified tumor-specific T lymphoblasts or NRL was mixed with equal parts Freund's complete adjuvant; 0.2 ml of the mixture containing 10^7 cells was injected intraperitoneally. Two booster injections of cells in Freund's incomplete adjuvant were given 3 and 6 weeks later. Ten days after the final immunization, the mice were challenged with 10^7 viable tumor cells injected subcutaneously or the immune reactivity of spleen cells from such mice was tested *in vitro*.

Chromium Release Assay. Cytotoxicity assays for cell-mediated cytolysis of tumor cells, tumor-specific lymphoblasts, or NRL were identical (19). About $5–10 \times 10^6$ target cells were labeled with 100 μCi (1 Ci = 3.7×10^{10} becquerels) of ^{51}Cr for 1 hr at 37°C. Samples (100 μl) of effector cells at various concentrations were placed in V-bottom 96-well microtiter plates (Cooke, Alexandria, VA), and mixed with 10^4 ^{51}Cr-labeled cells in 100 μl of complete medium for 3–4 hr at 37°C. At the end of the incubation, plates were spun at $800 \times g$ and 100 μl of supernatant was withdrawn. Spontaneous release was 10–15% of the maximum release of radiolabel during the incubation period for the fibrosarcoma lines and less than 25% of the maximum release for the purified lymphocytes. The percentage of specific lysis was calculated by the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

RESULTS

Specific Unresponsiveness to Tumor Cells *In Vivo*. Normal animals were repeatedly immunized with 1591-specific lymphoblasts in adjuvant, with NRL in adjuvant, or with adjuvant alone. Other control animals were either left untreated or were thymectomized and x-irradiated. Ten days after the last immunization, immunized and control animals were challenged with 10^7 viable 1591 or 1316 cells to test their resistance to the tumor.

We found that immunization with 1591-specific lymphoblasts selectively suppressed the resistance of the immunized mice to the 1591 tumor cells; i.e., these cells, which are regularly rejected by normal mice, now formed tumors that eventually killed the animals (Table 1). Identical results were obtained in separate experiments done several months apart. Various controls were performed to test for the specificity of the suppression. Immunization with 1591-specific lymphoblasts did not interfere with the resistance of these animals to 1316 tumor cells. Both 1591 and 1316 tumor cells were rejected by normal animals, animals immunized with adjuvant alone, or animals immunized with NRL that had been isolated from the same MLTC as the 1591-specific lymphoblasts. Immunization with nonspecific lymphoblasts generated in secondary cultures of spleen cells from mice injected with the variant line likewise did not render the animals susceptible to challenge with either the parental 1591 tumor cells or 1316 tumor cells. Animals that were nonspecifically immunosuppressed by adult thymectomy and x-irradiation all developed tumors, as expected, when challenged with either cell line.

Table 1. Specific suppression of resistance to tumor cells induced by immunization with syngeneic tumor-specific lymphoblasts

Immunogen	Tumor line injected*	Tumor incidence† Exp. 1	Tumor incidence† Exp. 2
1591-specific lymphoblasts	1591	3/4‡	4/6‡
	1316	0/5	0/5
NRL	1591	0/5	0/5
	1316	0/5	0/5
Anti-variant lymphoblasts§	1591	ND	0/5
	1316	ND	0/5
Freund's adjuvant alone	1591	0/5	0/5
	1316	0/5	0/5
Thymectomy, 500 rads	1591	ND	4/4
	1316	ND	4/4
No treatment	1591	0/10	0/10
	1316	0/10	0/10

ND, not done; 500 rads = 5 grays.

* Ten days after the third immunization 10^7 or 5×10^7 tumor cells were injected subcutaneously between the shoulders on the back of each of two or three animals. The results of both groups were identical and are therefore pooled in this table.

† No. of animals with progressive tumors per no. of animals in a group. All groups were monitored daily for tumor growth and size until three months after challenge. At this time, all tumor-bearing animals became moribund and had tumors up to 30 mm in large diameter. In animals that rejected the tumor, the tumors showed initial growth only up to 2 mm in large diameter and regressed during the subsequent days.

‡ Considering the group immunized with 1591-specific lymphoblasts and challenged with 1591 tumor cells and the group of any given control as independent random samples of 10, these groups are significantly different by Fisher's exact test at a p value of 0.002.

§ Anti-variant lymphoblasts were lymphoblasts isolated from day 5 secondary cultures of spleen cells and 1591 variant tumor cells.

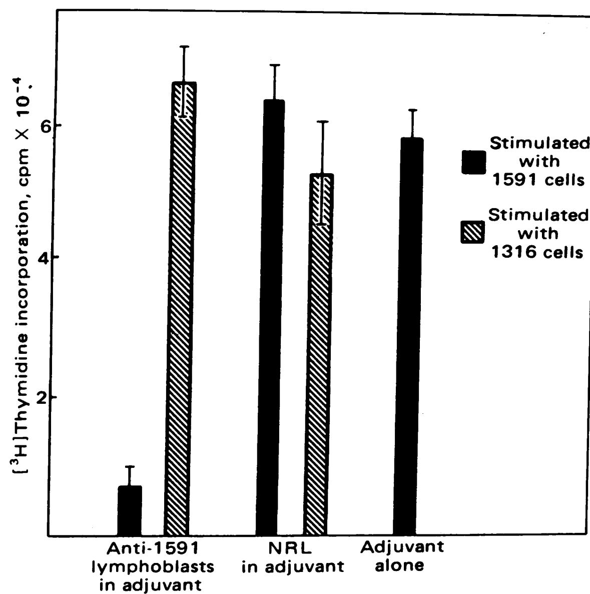

FIG. 1. Demonstration of unresponsiveness to 1591 tumor cells induced in C3H mice by immunization with syngeneic 1591-specific lymphoblasts. Responder cells were spleen cells from animals immunized three times with 1591-specific lymphoblasts, NRL, or adjuvant alone. About 7×10^5 spleen cells and 2×10^3 mitomycin C-treated tumor cells were incubated together for 72 hr. Then 1.0 μCi of [^{3}H]thymidine was added and the cell cultures were harvested 12 hr later. Error bars indicate ± 1 SD of four replicate cultures.

We tested whether the blast immunization created conditions that favored the outgrowth of progressively growing variants of the parental 1591 tumor cell lines. Thus, biopsies were performed when tumors measured 25 mm in largest diameter. Histologic sections of these biopsy materials revealed fibrosarcoma in all cases. Normal animals were then injected with either fragments from the biopsy material or cultured tumor cells after cells from the biopsies had been readapted to growth *in vitro*. It was found that the animals regularly rejected both tumor fragments and cultured tumor cells from the biopsies.

Specific Unresponsiveness to Tumor Cells *In Vitro*. We have attempted to characterize the immunologic alterations in the lymphoblast-immunized animals by using *in vitro* assays. Therefore, spleen cells from lymphoblast-immunized animals were tested for their response to 1591 or 1316 tumor cells *in vitro* in a primary MLTC. Spleen cells were stimulated with either 1591 or 1316 tumor cells and their response to both lines was determined by measuring the amount of [^{3}H]thymidine incorporation. Fig. 1 shows that spleen cells of 1591 blast-immunized animals were unresponsive to 1591 cells, whereas spleen cells from the same animals were stimulated by 1316 tumor cells. Furthermore, those cultures containing cells from blast-immunized animals and 1591 tumor cells failed to develop cytolytic T cells against 1591 tumor cells (<1% specific lysis at a 100:1 effector-to-target cell ratio). In contrast, spleen cells from the same animal, but stimulated with 1316 tumor cells in a primary MLTC, showed significant lysis of 1316 tumor cells (>25% specific lysis at a 100:1 effector-to-target cell ratio). Spleen cells from NRL-immunized or normal animals generated specific cytolytic T cells to both tumor cell lines equally well.

Because blast-immunized animals failed to respond to 1591 tumor cells, we examined whether these animals might have developed autoimmunity to 1591-specific lymphoblasts as a result of immunization with these cells. Thus, 1591-specific lymphoblasts isolated from secondary MLTCs of 1591-immunized animals were labeled with chromium and used as targets; spleen cells of the blast-immunized animals were used as effectors. Table 2 shows that animals immunized with 1591-specific lymphoblasts developed cytolytic spleen cells that effectively lysed 1591-specific lymphoblasts. The cytolytic activity of these cells was selective for 1591-specific lymphoblasts, because 1316-specific lymphoblasts were not affected. This killing potential of spleen cells was completely eliminated by anti-Thy 1.2 antiserum and complement. These results suggest the presence of anti-idiotypic T cells specific for anti-1591 lymphoblasts in the anti-1591 lymphoblast-immunized animals. The induction of anti-idiotypic killer cells specific for 1591-specific lymphoblasts has been observed repeatedly. Not 1 of over 30 individually tested blast-immunized animals has failed to show this specific cytolytic activity.

Analysis of Spleen Cells from Blast-Immunized Animals that Rejected the Tumor. Although immunization with tumor-specific lymphoblasts induced specific suppression of tumor resistance in most of the animals, three of ten blast-immunized animals rejected the challenge with the 1591 tumor

Table 2. Demonstration of cytolytic "anti-idiotypic" T cells in blast-immunized mice

Effector spleen cells from animals immunized with*	Animal	Specific lysis of target cells, %† — Anti-1591 blasts‡, 250:1, C alone	250:1, Anti-Thy 1.2 and C	100:1, C alone	100:1, Anti-Thy 1.2 and C	Anti-1316 blasts 250:1	Anti-1316 blasts 100:1
1591-specific lymphoblasts	1	59	<0	16	<0	<0	<0
	2	51	0	18	<0	<0	<0
NRL	1	<0	<0	<0	<0	<0	<0
	2	<0	<0	<0	<0	4	<0
Adjuvant alone	1	<0	<0	<0	<0	2	<0
	2	<0	<0	<0	<0	1	<0

* Effector spleen cells were obtained from C3H mice immunized three times with 10^7 1591-specific lymphoblasts in adjuvant, 10^7 small NRL in adjuvant, or adjuvant alone. Spleens were removed and tested 10 days after the last immunization.

† Cells were tested in a 4-hr ^{51}Cr release assay using as target purified 1591-specific or 1316-specific lymphoblasts at a 250:1 or 100:1 effector-to-target cell ratio. These lymphoblasts were obtained after *in vitro* restimulation of 1591 or 1316 immune spleen cells and purified by equilibrium density centrifugation.

‡ T cells were eliminated by incubating 2.5×10^7 spleen cells in 1 ml of a 1:50 final dilution of anti-Thy 1.2 antiserum (Litton Bionetics, Kensington, MD) for 30 min on ice. Cells were then washed and exposed to a 1:5 final dilution of guinea pig complement (C) for 45 min at 37°C. This procedure killed approximately 45% of the spleen cells as determined by trypan blue exclusion, and the remaining cells were greater than 95% Ig-positive.

(Table 1). The failure of these blast-immunized animals to accept the tumor graft could have been caused by the ineffectiveness of either the blast immunization or the tumor challenge. It was, therefore, of interest to analyze whether these animals could produce cytolytic T cells specific for 1591 tumor cells *in vitro*. Consequently, spleen cells from two blast-immunized animals that had rejected the tumor challenge 5 months earlier were cocultured with 1591 tumor cells in a MLTC. Table 3 shows that spleen cells from the blast-immunized tumor-resistant animals exhibited the same capability to specifically lyse the tumor cells at an effector-to-target cell ratio of 50:1 as spleen cells from NRL-immunized or normal animals that had also rejected the tumor challenge 5 months earlier.

These results show that the blast immunization was ineffective in inducing specific unresponsiveness to 1591 tumor cells in these two animals. It is nevertheless conceivable that the blast immunization induced anti-idiotypic immunity that effectively eliminated the normally predominant tumor-reactive lymphocyte clone(s) and that after the tumor cell challenge new, previously silent, clones of tumor-specific lymphocytes developed in these animals. We therefore determined whether the 1591 tumor-specific lymphoblasts from the blast-immune, tumor-resistant animals, when used as ^{51}Cr-labeled targets, could be lysed by the "anti-idiotypic" killer T cells of blast-immunized animals. The results in Table 4 show that anti-idiotypic effector spleen cells could effectively lyse "control" target cells consisting of 1591 tumor-specific lymphoblasts from the normal or NRL-immunized tumor-resistant animals. In contrast, 1591 tumor-specific lymphoblasts from the blast-immune tumor-resistant animals were completely insensitive to lysis, indicating that the normally predominant lymphocyte clone(s) had been effectively eliminated in these animals.

DISCUSSION

We have found that immunization with 1591 tumor-specific lymphoblasts induces an autoimmune reaction to these cells and also induces unresponsiveness to 1591 tumor cells *in vivo* and *in vitro*. Several lines of evidence suggest specificity in the observed effects. Resistance of the 1591 blast-immunized animals to 1316 tumor cells *in vivo* and responsiveness of the spleen cells from these animals to 1316 tumor cells *in vitro* were unimpaired. In agreement with this is the finding that the spleen cells from the 1591 tumor-specific lymphoblast-immunized animals did not kill 1316 tumor-specific lymphoblasts. Furthermore, immunization with NRL or with lymphoblasts not having specificity for 1591 tumor cells was ineffective in inducing the observed effects.

Table 3. Capability of spleen cells from the blast-immunized tumor-resistant animals to respond to 1591 tumor cells *in vitro*

Exp.	Responder spleen cells from animals pretreated with*	Specific lysis of target cells, %†	
		1591 tumor cells	1316 tumor cells
1	Blast immunization	26	0
	NRL immunization	24	2
2	Blast immunization	16	<0
	NRL immunization	13	<0

* The two 1591-specific lymphoblast-immunized animals that subsequently rejected 1591 tumor cells, and two control animals immunized with NRL, which also subsequently rejected 1591 tumor cells, are included. Five months after the challenge with tumor cells, spleen cells of these animals were stimulated in 5-day MLTCs with 1591 tumor cells and tested in ^{51}Cr-release assays.

† Cells were tested in a 3-hr ^{51}Cr release assay using an effector-to-target cell ratio of 50:1.

Strong evidence indicating idiotype-specific immune reactions to tumor-specific lymphocytes comes from the analysis of animals that rejected the tumor in spite of the blast immunization. These animals exhibited the same capability to specifically lyse tumor cells as control animals, yet they responded by generating 1591 tumor-specific lymphoblasts that were completely insensitive to lysis by anti-idiotypic effector cells. This is in contrast to the large number of control animals, which regularly responded by generating tumor-specific lymphocytes sensitive to anti-idiotypic effector cells. Therefore, these findings suggest that autologous idiotype-specific immune reactions were induced by the immunization and this immunity eliminated the animal's own normally predominant tumor-reactive lymphocyte clone(s). Our findings also suggest that idiotypically different, previously undetected, lymphocyte clones developed subsequent to antigenic challenge with the tumor cells in the two blast-immunized animals that resisted the tumor challenge. It has been suggested by experiments in nontumor systems (20) that antigenic stimulation is important in the development of new clones of lymphocytes.

Our experiments show that blast-immunized animals develop anti-idiotypic cytolytic T cells that can eliminate tumor-specific lymphocytes. This observation suggests one of the mechanisms by which the unresponsiveness might have been induced by the blast immunization. We have consistently observed the induction of anti-idiotypic immunity in a large number of animals, and it appears that blast-immunized animals could reject the tumor challenge only if they developed idiotypically different tumor-specific lymphocyte clones. We have not as yet determined whether anti-idiotypic antibodies are present in the serum of blast-immunized animals.

Although we have not yet extended our findings to another tumor system, the results of this study are consistent with our hypothesis that changes in the balance of tumor-specific and anti-idiotypic lymphocyte clones may critically influence the capability of an individual to respond to tumor antigens. Such changes may be the result of chronic antigenic stimulation, which has been shown to induce anti-idiotypic immunity (21–23). It is therefore conceivable that anti-idiotypic immune

Table 4. Failure of tumor-specific lymphoblasts from the blast-immunized tumor-resistant animals to be lysed by "anti-idiotypic" immune cells

Exp.*	Specific lysis of target cells, %*			
	1591-specific lymphoblasts from			1316-specific lymphoblasts from
	1591 tumor-immune	Blast-immunized 1591 tumor-immune	NRL-immunized 1591 tumor-immune	1316 tumor-immune
1	68	0	42	1
2	37	1	54	7

* Effector cells were "anti-idiotypic" T cells obtained from spleens of animals immunized three times with 1591 tumor-specific lymphoblasts 1 month earlier. Results are given as percent specific lysis in a 4-hr ^{51}Cr release assay at a 250:1 effector-to-target cell ratio.

† Target cells were ^{51}Cr-labeled lymphoblasts from the two 1591-specific lymphoblast-immunized tumor-resistant animals (see Table 1, exp. 2) or from two NRL-immunized tumor-resistant animals of the same experiment. The lymphoblasts were obtained after a 5-day MLTC with 1591 tumor cells. The specificity of the lymphocytes for 1591 tumor cells is shown in Table 3. Control target cells were ^{51}Cr-labeled tumor-specific lymphoblasts from spleens of normal animals injected with either 1591 or 1316 tumor cells restimulated in a 5-day MLTC with the corresponding cell line. Tumor-specific lymphoblasts were separated from nonresponding lymphocytes by equilibrium density centrifugation before labeling.

responses are induced during tumor development. Our findings also indicate the problem that anti-idiotypic immunity might be induced when tumor-specific antibodies or T cells are passively transferred during immunotherapy. Under such conditions, this treatment would obviously be disadvantageous to the host.

It has been demonstrated that anti-idiotypic immunity may be preexisting in normal animals (24), and that elimination of such anti-idiotypic lymphocyte clones by the use of anti-(anti)-idiotypic immunoreagents can increase the immune responses of certain clonotypes (25). Furthermore, anti-idiotypic reagents can, under defined conditions, stimulate the lymphocyte clones expressing the idiotype (6, 26), even in the absence of antigen (27). Thus, anti-idiotypic and anti-(anti)-idiotypic immunoreagents may become very useful tools to specifically stimulate immune responses to tumor antigens.

We gratefully acknowledge the expert technical assistance of Ms. Connie Philipps. Furthermore, we thank Dr. J. Quintans for his critical review of the manuscript. This research was supported by U.S. Public Health Service Grants 1-RO1-CA-22677 and CO-75380 from the National Cancer Institute and by Grant IN-41-R from the American Cancer Society. P.M.F. is supported by National Research Service Award NOI-T32-AI-7090 and H.S. is the recipient of Research Career Development Award CA-00432 from the National Institutes of Health.

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