Effects of Immunoactive and Cytotoxic Drugs on Cultured Rat Myeloma Cells.

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Introduction

The IR162 rat myeloma is a transplantable IgE-secreting tumor which was derived from a spontaneous tumor in a LOU/C rat (3). It has been established in cell culture as the Ta4 line (10, 11). Ta4 cells are tumorigenic in LOU/C and LOU/M rats, and they secrete IgE *in vitro*. The IgE produced by tumor cells is readily monitored in serum or in tissue culture fluid (11). The IR162 rat myeloma therefore provides a model system for assessment of putative therapeutic agents that might be of benefit in human myeloma. Compounds may be examined for inhibition or for stimulation of Ta4 growth, and for IgE synthesis *in vitro*. These compounds may then be evaluated *in vivo*.

Three classes of compounds have been chosen for evaluation *in vitro*. Cytotoxic agents were examined in Ta4 cells to determine whether they were lytic, and if there were a differential effect on cell growth and on secretion of a specific protein, IgE. There is ample evidence that the immune system can play a part in cancer therapy (5), and certain mouse plasmacytomas have been subjected to regulation by the immune system (1,20). Therefore a number of immunomodulating drugs were tested for their direct effect on Ta4 cells, which are of B lymphocyte origin (3). Finally, three compounds were examined which might help to elucidate the mechanisms of inhibition of Ta4 cells.

Materials and Methods

The Ta4 cells were propagated in stationary suspension cultures in RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 10 percent fetal calf serum. Passages were made twice weekly by pipetting to disperse cell clumps. The cells were diluted 1:10 in fresh medium. Late log phase cells were used in all experiments.

Cell growth was determined on the basis of the change in total cell protein using the Lowry protein assay as adapted for cell culture by Holden et al (17) or by cell counts using a Model ZBI Coulter counter. A total protein assay was initially chosen because of my interest in evaluating secreted protein. However, parallel determinations using total protein and cell counts gave similar results.

The IgE content in tissue culture media was determined by quantitative passive hemagglutination inhibition (HAI) (11). Briefly, anti-rat IgE antiserum (Miles Laboratories) was diluted serially in 96 well microtiter plates to which diluent or diluted cell culture medium was added. The plates were agitated and incubated at room temperature for 30 minutes. IgE-labelled sheep red blood cells were added; plates were agitated and incubated overnight; and the extent of hemagglutination inhibition was determined. All determinations were performed in triplicate.

Dose response curves were performed by adding compounds to triplicate samples of cell culture medium in which Ta4 cells were grown for 72 or 96 hours in order to establish tissue culture inhibitory dose 50's (ID50) for both cell growth and IgE synthesis. In order to calculate the ID50, growth or IgE content was plotted

versus the logarithm of the drug concentration, and linear regression was used on the linear portion of the curve. Since HAI titers depend on twofold serial dilutions, they are not as precise as protein determinations or cell counts. Therefore, cell growth and IgE synthesis were not considered to be differentially affected unless their respective ID50's exhibited more than a twofold difference. These experiments have been repeated with Ta4 cells in culture over a period of three years; and the cell line appears to be quite stable in its response to chemical agents.

The following agents were evaluated: cycloheximide (Sigma Chemical), theophylline (Sigma Chemical), aminophylline or (theophylline)$_2$EDTA (Sigma Chemical), isoproterenol (K and K Laboratories), phytohemagglutinin M (Difco Laboratories), concanavalin A (Miles Laboratories), lipopolysaccharide (Difco Laboratories), pokeweed mitogen (Grand Island Biological), 5-bromo-2-deoxyuridine (Calbiochem), idoxuridine (Sigma Chemical), 8-azaguanine (Sigma Chemical), azathioprine (Burroughs Wellecome), estracyt (A.B. Leo, Halsingborg, Sweden), methotrexate (Sigma Chemical), cyclophosphamide (Mead Johnson), poly (I):poly (C) (Miles Laboratories), poly (A):poly (U) (Miles Laboratories), levamisole (Sigma Chemical), tilorone (Merrill-National Laboratories).

TABLE 1. *Drug Toxicity Levels*

Compound	Cell Growth ID50 (ug/ml)#	IgE Production ID50 (ug/ml)	Range (ug/ml)	Number of Experiments
Mitogens				
Phytochemagglutinin-M	>1/10* No effect	HAI Unreliable	1/10000-1/10	3
Concanavalin A	>50 No effect	HAI Unreliable	0.1-50	3
Endotoxin	>100 No effect	>100 No effect	0.1-100	2
Pokeweed Mitogen	>1/10* No effect	>1/10 No effect	1/10000-1/10	1
Base Analogs				
Bromodeoxyuridine	0.23	4.5	0.01-100	6
Idoxuridine	2.6	10	0.01-100	2
8-Azaguanine	16	ND	0.1-100	3
Azathioprine	53	100	0.1-100	2
Anticancer Agents				
Estracyt	36	22	0.1-50	1
Methotrexate	0.0029	ND	0.0005-50	3
Clclophosphamide	500	ND	0.1-500	1
Not activated	>500	ND	0.1-500	1
Activated	9	6	0.1-50	5
Immunostimulators				
PolyI:polyC	>100 No effect	>100 No effect	0.1-100	4
PolyA:polyU	>100 No effect	>100 No effect	0.1-100	4
Levamisole	340	240	0.1-1000	3
Tilorone	1.8	3.0	0.1-100	5
Others				
Cycloheximide	0.05	0.085	0.01-50	2
Theophylline	>100	>100	0.1-100	3
Isoproterenol	27	234	0.1-100	4

Data are expressed as the mean of determinations from several experiments.

* Since these compounds are not sold by weight, dilutions were used. Generally they are used at a 1/100 dilution for effective mitogenesis.

Results and Discussion

Comparisons of growth and IgE secretion yielded three patterns. Compounds such as the polynucleotides, mitogens, and theophylline, had no effect on either cell growth or IgE synthesis over the range tested. (Table 1). That range included those concentrations which were shown to be active in other systems. Most other compounds inhibited growth and IgE synthesis to approximately the same extent. At lower doses IgE synthesis and growth were equivalent for concanavalin A (Con A) and phytohemagglutinin M (PHA). At higher drug concentrations, HAI results were considered unreliable due to the agglutinating effects of these compounds.

Only the base analogs 5-bromo-2-deoxyuridine (BUDR) and 5-iodo-2-deoxyuridine (IUDR) produced a differential effect on cell growth and IgE secretion. Both drugs inhibited cell growth at significantly lower concentrations than IgE synthesis. The immunosuppressive purine analog azathioprine had a similar but less pronounced effect.

Cell lysis was occasionally observed at high drug concentrations but not at dosages at which the compounds are normally used. In confirmation of previous reports the release of IgE upon cell lysis was very small, and the IgE levels remained stable throughout the course of the experiments (10).

Since one of the goals of this work was to establish an *in vitro* model which would predict *in vivo* results, cycloheximide, theophylline, and isoproterenol were tested to characterize the system. Cycloheximide inhibits protein synthesis at the ribosomal level (7). It should therefore inhibit cell growth and IgE synthesis to approximately the same extent, and this was the case.

Two agents which increase cyclic adenosine monophosphate levels were tested because of the reports of the affects of cyclic nucleotides on immune cell function (15, 18, 24). Theophylline had no effect on Ta4 cells, either when it was used as theophylline or as aminophylline. Isoproterenol was equally toxic to growth and IgE synthesis.

The alkylating agent cyclophosphamide (CPA) is a phase nonspecific cytotoxic agent which is used in the chemotherapy of multiple myeloma (8). It has been shown to effectively reduce the tumor burden in rats bearing subcutaneous IR162 rat myelomas (10). CPA must be activated by the liver *in vivo* (8). The ID50 of CPA before activation was greater than 500 ug/ml. However, when rats were injected with 500, 200, and 100 mg/kg CPA and bled at one hour, at which time serum levels of the active metabolite are near maximum (30), the ID50 was 9 ug/ml and the dose response was essentially linear. Thus Ta4 cells respond similarly to human myeloma (8), and this assay may be used for serum drug levels. Methotrexate is a phase specific agent which acts as an antimetabolite and is commonly used for hematopoietic malignancies (23). Ta4 cells were sensitive to methotrexate, and therapy trials indicated the value of this agent *in vivo* against rat myeloma (Manuscript in preparation).

Cytoreductive therapy of multiple myeloma is generally only effective in reducing the tumor burden; spontaneous remissions are unknown; and patients are generally severely immunosuppressed (8). Human myeloma therefore appears to be a candidate for immunotherapy, and the rat model has potential for immunotherapy trials. Passive spleen cell transfer has been shown to prolong survival and has resulted in some cures (2), and Ta4 cells are sensitive to antibody and complement mediated lysis (unpublished). In certain mouse plasmacytomas the immunoglobulin acted as an important antigen in tumor rejection (13), and the extent of cell differentiation and immunoglobulin secretion was immunologically mediated.

Therefore, a number of compounds, which have been reported to have effects on cells of the immune system, were tested.

Neither the T-cell mitogens, PHA and Con A, nor the B-cell mitogens, pokeweed mitogen and endotoxin, had any effect on the tumor cells (14, 29). Mitogens were evaluated because of the lymphocyte origin of the Ta4 cells, although their state of differentiation is beyond that at which mitogens are generally active. Endotoxin was of particular interest because of its reported necrotic activity against tumors, which has been attributed to the production of tumor necrosis factor *in vivo* (16).

The double stranded polyribonucleotides have been reported to inhibit tumor growth in several rodent models (16, 19, 26). Neither was active against Ta4 cells. However, poly(I):poly(C) is a potent inteferon inducer in rodents (12), and the Ta4 cels show some sensitivity to rat interferon (unpublished). This agent may have some promise for immunotherapy. Tilorone (9, 21) and levamisole (6, 16, 25, 27) have been reported to be immunoactive and to be beneficial in certain cancers. Neither compound stimulated the growth of Ta4 cells; actually cell growth and IgE synthesis were equally inhibited by the drugs. Therefore, the polyribonucleotides, tilorone, and levamisole might be candidates for *in vivo* immunomodulation. They can be used in animals, but not at dosages which would reduce the tumor burden; and they do not directly stimulate the tumor cells.

BUDR and its related pyrimidine analog IUDR both inhibited the Ta4 cell growth at much lower concentrations than IgE synthesis. In fact at concentrations near the ID50 IgE synthesis proceeded at control levels (no drug added) for two or three days after cell growth ceased. These agents may have potential for sensitizing the tumor cells to immune killing. Certain metabolic inhibitors have been shown to alter the sensitivity of tumor cells to immune attack (28). These compounds also illustrate that the secretion of immunoglobulin by Ta4 cells is subject to modulation other than growth inhibition. Perhaps this is related to increased differentiation induced by BUDR in cultured neuroblastoma cells (4).

In conclusion the Ta4 cells have been used to evaluate several cancer chemotherapeutic agents and putative immunotherapeutic agents for their effects of growth and IgE synthesis. Most compounds produced equivalent effects on both parameters, but BUDR and IUDR inhibited growth while allowing continued IgE production. The assay has potential for screening putative chemotherapeutic agents for myeloma, and it can be used to assay serum levels of drugs to which the cells are sensitive.

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