Stimulation of Cells by Antibody

Abstract. *Tumor cell lines exposed to immunoglobulins specific for cell surface antigens developed increased cellular incorporation of [^{125}I]iododeoxyuridine and [^{3}H]thymidine (up to 200-fold increases over cells treated with normal rabbit immunoglobulins). Antibody-stimulated cells multiplied more rapidly and lived longer than control cells in tissue culture. These observations were made both with cells substituted with 2,4,6-trinitrophenol and purified antibody against 2,4,6-trinitrophenol, and with several cell lines and their respective whole-cell antibodies. Antibodies that were stimulatory at low concentrations were cytotoxic at high concentrations. These observations may have significance in regard to enhancing effects of antibodies on tumor cell growth in vivo.*

Prehn developed a theory of immunostimulation of tumor cells which proposes that both cellular and humoral immune factors may facilitate tumor growth (*1*). The immune effect proposed by Prehn is biphasic; a minimal immune response to tumor cells accelerates tumor cell growth, whereas a vigorous immune response is cytotoxic. The possibility that antibodies might stimulate tumor cell growth is not unreasonable in view of the capacity of lymphocytes to undergo blast transformation with concomitant increased DNA synthesis after exposure to antibodies against immunoglobulins and against lymphocytes (*2*). However, lymphocytes are programmed to respond to surface perturbation by antigen while nonlymphoid cells are not. While studying cytotoxic effects of antibody-enzyme conjugates on tumor cells in vitro (*3*), we observed that cells exposed to antibody exhibited increased incorporation of the radioactive DNA precursors [^{125}I]iododeoxyuridine ([^{125}I]IdU) and [^{3}H]thymidine ([^{3}H]dT) into DNA (*4*). We now present evidence that the phenomenon occurs with several cell lines, is antibody-mediated, and involves increases in cell number as well as alterations in nucleoside incorporation into DNA.

The cell lines studied included HeLa, HEp-2 (both from American Type Culture Collection), mouse L-929 (Grand Island Biological), HT-29 (human colonic cancer), MOPC-315 (mouse plasmacytoma), and cells (HeLa, HEP-2, and L-929) substituted with 2,4,6-trinitrophenyl (TNP) groups (*3*). Antiserums were obtained by immunization of randomly bred rabbits with 2×10^6 washed tumor cells or TNP-substituted bovine gamma globulin. Cultured cells were scraped off a monolayer (except free-floating MOPC-315), washed three times in Hanks buffered salt solution (HBSS), suspended in HBSS, and emulsified with an equal volume of Freund's complete adjuvant (Difco). The rabbits were immunized twice with cells in complete Freund's adjuvant and once with cells in HBSS at 2-week intervals. Blood was collected at weekly intervals thereafter. Rabbit antiserum against L cells (10 ml) was absorbed for 1 hour at room temperature by 1 ml of a packed crude cell homogenate of liver, spleen, kidneys, heart, and skeletal muscle of a C3H mouse. Rabbit antiserums against HeLa and HEp-2 were similarly absorbed successively with 10^8 heterologous cells (HEp-2 or HeLa, respectively) and with 10^8 HT-29 cells. Rabbit antiserum against MOPC-315 was absorbed with Balb/c tissue. Serums were heat-inactivated for 30 minutes at 56°C and sterile-filtered. Control serums were prepared in identical manner (but without cells) and were treated similarly. Antibody against TNP was specifically purified from antiserums against TNP from hyperimmunized rabbits as described by Little and Eisen (*5*), and was at least 92 percent pure as judged by precipitation studies with TNP-substituted bovine serum albumin.

HeLa, HEp-2, and L cell lines were maintained in Eagle's minimum essential medium (MEM) plus 10 percent fetal calf serum (NM, nutrient medium) at 37°C in a humidified atmosphere of 95 percent air and 5 percent CO_2. Cells substituted with TNP groups were prepared by reaction with TNP-sulfonic acid (*3*). Unreacted or TNP-substituted cells were suspended in MEM containing 10 percent heat-inactivated fetal calf serum (hNM), and a small volume of phosphate-buffered saline (PBS) containing appropriate reagents was added (PBS, diluted antiserum, diluted normal serum, purified antibody against TNP, or normal rabbit gamma globulin at similar protein concentrations). In experiments with antibody against TNP, incubations with ε-dinitrophenyllysine (ε-DNP-lysine) were also performed to establish that the response to the antibody was specifically inhibitable by hapten. After incubation for 30 minutes at 37°C, the cells were washed three times with hNM and cultured in individual tubes (12 by 75 mm, Falcon Plastics) at initial cell densities of 2.5 to 10×10^4 per milliliter for up to 5 days. In some experiments, reagents remained in culture tubes throughout the experiment. At selected intervals, [^{125}I]-IdU (Amersham) or [^{3}H]dT (New England Nuclear) was added to each tube. After an additional 24 hours of incubation, the cells were harvested. Excess radioactivity was removed by repeated

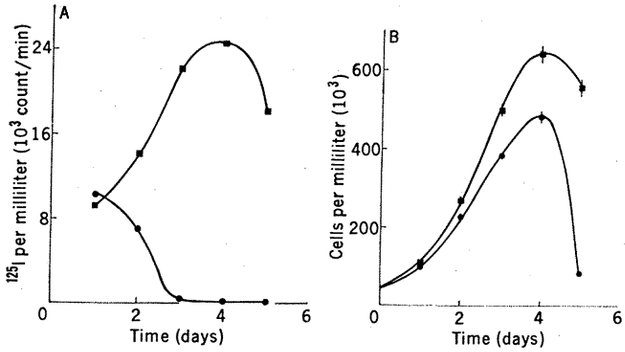

Fig. 1. (A) Incorporation of [^{125}I]iododeoxyuridine into L-929 cells treated with antiserum against L cells or normal rabbit serum. Dots represent L cells incubated in hNM with normal rabbit serum at 1 : 200 final dilution; squares represent L cells incubated in hNM with antiserum against L cells at 1 : 200 final dilution. Values are radioactivity of [^{125}I]IdU for successive 24-hour periods; total radioactivity concentration was 0.05 μc/ml. Results are the mean of triplicate determinations; standard error of means fell within the range occupied by symbols. For days 2 to 5, $P < .001$. (B) Viable cell counts of L-929 cells treated with antiserum against L cells or normal rabbit serum; serum dilutions and symbols as in (A). Values are the mean of triplicate cell counts performed with a hemacytometer (two fields averaged per count) ± standard error of the mean (brackets). The differences in cell counts became highly significant (day 1, $P > .3$; day 2, $.02 < P < .05$; day 3, $.001 < P < .01$; and days 4 and 5, $P < .001$). To facilitate cell counting, cell pellets were washed in HBSS free of calcium and magnesium, treated with 0.05 percent trypsin for 5 minutes, and counted in hNM containing 0.01 percent nigrosine. The abrupt fall in cell numbers of control cells on day 5 was caused by cell crowding and lack of fresh medium, conditions that did not similarly affect the antibody-treated cells.

Table 1. [^{125}I]Iododeoxyuridine and [^{3}H]thymidine uptake in TNP-substituted L-929 cells treated with antibody against TNP for 30 minutes. Cells were labeled with [^{125}I]IdU (0.04 μc/ml) or [^{3}H]dT (0.50 μc/ml) during the period between 48 and 72 hours after exposure to antibody. Values are means of triplicate samples ± standard error; γG, gamma globulin.

Conditions	^{125}I in cell pellets (count/min)	^{3}H in cell pellets (count/min)
TNP–L cells (50,000 per ml)	1,023 ± 87	15,900 ± 1,612
TNP–L cells + antibody to TNP (7 μg/ml)	24,128 ± 345*	242,296 ± 3,839*
TNP–L cells + antibody to TNP (7 μg/ml) + DNP-lysine ($10^{-4}M$)	770 ± 85	13,849 ± 896
TNP–L cells + rabbit γG (7 μg/ml)	926 ± 20	14,504 ± 1,171

* Significantly different ($P < .001$) from control values (line 1). All other values not statistically different ($P > .1$).

centrifugation and washing with hNM followed by precipitation with 6 percent trichloroacetic acid. Insoluble radioactivity was determined by gamma (^{125}I) or liquid scintillation (^{3}H) counting.

A 30-minute exposure of TNP-substituted L cells to antibody against TNP followed by washing stimulated the incorporation of [^{125}I]IdU and [^{3}H]dT approximately 20-fold over that of control cells (Table 1). Practically identical results were obtained in an experiment in which all reagents remained in the culture medium during the entire experiment. The hapten inhibitor ε-DNP-lysine ($10^{-4}M$) prevented the immune stimulation of nucleoside uptake. "Nonspecific" rabbit gamma globulin at the same protein concentration as the purified antibody against TNP showed no stimulating effect on radioactivity uptake into TNP-substituted L cells. A parallel experiment (data not shown) with unsubstituted L cells did not reveal any differences in incorporation for the four conditions in Table 1. The differences in nucleoside incorporation between control TNP-substituted cells and those treated with antibody against TNP were maximal 48 to 96 hours after initial exposure to antibody.

The response with small amounts of highly purified antibody against TNP and the inhibition of the response by hapten left no doubt that the stimulation of [^{125}I]IdU and [^{3}H]dT uptake was immunologically mediated. We next established that the immune stimulation phenomenon could also be elicited in unsubstituted cells by using antibodies to "natural" cell antigens. Rabbit antiserums against tumor cells and control rabbit serums were tested for capacity to stimulate nucleoside incorporation into homologous and heterologous cell lines. Cells were treated with trypsin for 2 minutes, washed with hNM, placed in tubes, and incubated with various reagents (PBS, control rabbit serum, or immune rabbit serum) for either 30 minutes or for the entire experiment (up to 5 days). In addition to measurements of radioactive nucleoside uptake, cell counts were performed after each 24 hours of incubation (legend to Fig. 1).

The incorporation of [^{125}I]IdU into L cells at various times after exposure to antiserum against L cells or to normal rabbit serum (each at a final dilution of 1 : 200) is shown in Fig. 1A. For the first 24 hours, the rates of incorporation of L cells treated with antibody and those treated with control serum were approximately equal, but in successive 24-hour periods the amounts of [^{125}I]IdU incorporated by the control cells progressively fell, whereas in cells exposed to antibody the amounts of [^{125}I]IdU (presumed DNA synthesis) rose to a maximum at 4 days. The ratios of absolute counts incorporated into antibody-stimulated cells to those for control cells are 0.89, 20, 52.5, 115, and 201 on days 1 through 5, respectively. The differences from days 2 to 5 are significant ($P < .001$).

Cell counts of a simultaneous duplicate experiment (Fig. 1B) indicate that the antibody-treated cells grew faster than the control cells from the outset and that by day 2 the differences had become highly significant (day 1, $P > .3$; day 2, $.02 < P < .05$; day 3, $.001 < P < .01$; and days 4 and 5, $P < .001$). Antibody-treated cells also differ from control cells in that they appear agglutinated and take more agitation to disperse.

Several lines of evidence suggest that the stimulating factor in the rabbit antiserum is the antibody per se. (i) Little if any stimulation was obtained with control serum. (ii) A dose-response stimulation curve was obtained by varying concentrations of antiserum. For example, values for [^{125}I]IdU incorporation of L cells in the period between 72 and 96 hours after incubation with 1 : 20, 1 : 200, and 1 : 2000 dilutions of antiserum against L cells were, respectively, 0.02, 101,, and 4.7 times those of L cells incubated with similar dilutions of normal rabbit serum. The 1 : 20 dilution of antiserum against L cells was toxic to the cells (as confirmed by killing effect in T flasks), whereas the 1 : 200 dilution stimulated cells to incorporate [^{125}I]IdU and divide. The 1 : 2000 dilution caused modest stimulation. (iii) In experiments in which cells were exposed to antibody for 30 minutes, previous prolonged trypsin treatment of the cells reduced the capacity of the antiserum to stimulate nucleoside incorporation. Presumably this is due to the removal of antigens from the cell surface by trypsin. (iv) The stimulatory effect could be removed by absorption of the antiserum with homologous tumor cells. (v) There was partial although not complete immunologic specificity in the immunostimulation pattern with different antiserums. The rabbit antiserums to HeLa, HEp-2, and L cells showed complete cross-reactivity with respect to stimulating these three cell lines but were unable to stimulate nucleoside incorporation in HT-29 or MOPC-315. Absorption of an antiserum against L cells with L cells reduced the capacity of that antiserum to stimulate L cells, HEp-2, and HeLa by approximately the same degree. Rabbit antiserum against MOPC-315 stimulated the homologous cell line to incorporate [^{125}I]-IdU (maximum twofold increase compared to control serum) and to increase in cell number (30 percent more than control serum) but did not stimulate any other cell lines (*6*). Rabbit antibody against HT-29 did not stimulate any cell line including HT-29. (vi) A stimulatory effect was obtained with only 1 μg of highly purified antibody against TNP or with as little as 20 μg of the gamma globulin fraction or 50 μg of the 7*S* immunoglobulin G fraction (only amount tested) of antiserum against L cells. (vii) Complement-mediated cytotoxicity was observed with L cells and both antiserum and gamma globulin against L cells but not with L cells and control serum and control gamma globulin (*6*). (viii) Finally, L cells exposed to antibody against L cells for 30 minutes displayed a patchy surface fluorescent staining pattern in the presence of a second antibody (fluorescein-conjugated goat antibody against

rabbit immunoglobulin), whereas L cells treated with normal rabbit serum showed no fluorescence (*6*). Further immunofluorescence studies showed the presence of cell-bound rabbit immunoglobulin with all of the cell-antiserum combinations with which stimulation occurs.

Other studies (*6*) indicate that the large increases in radioactive nucleoside uptake may be due in part to increased nucleoside transport, but it is clear from the measurements of cell number that cell proliferation is also being stimulated. There is a close analogy here to the stimulatory effects of lectins on lymphocyte cell growth, in which there are marked increases in radioactive [^{3}H]dT uptake with smaller increases in cell number and total cellular DNA content.

Our tentative conclusions are that antibodies specific for cell surface antigens can induce the cell to undergo DNA synthesis and cell division and that the antigenic determinants involved may be similar on the several different cell lines that show immunologic cross-reactivity. One of these cell lines, L-929, was transformed by a chemical carcinogen, methylcholanthrene, yet shows cross-stimulation with antiserums against two human cancers that have no known transforming agent. In cell lines stimulated by the same antiserums, the cross-stimulation we see could be due to the presence of common, tumor-related glycopeptides, but the possibility of viral or mycoplasmal infection of our cultures should also be considered. Viral infection is highly unlikely because we have obtained comparable immune stimulation with fresh cell lines in another laboratory with different equipment. Mycoplasma-free and mycoplasma-infected cell lines yield similar immune stimulation.

This is, to our knowledge, the first direct evidence that antibodies can stimulate the growth of transformed, nonlymphocytic cell lines. While the mechanism is unclear, there has been considerable speculation about the possible role of increased transport of essential nutrients into the cell during accelerated cell growth. In this connection, it is of interest that stimulation of active transport of potassium has been demonstrated in sheep LK red blood cells after incubation with sheep antibody against the L determinant (*7*). The possible relevance of these observations to blocking effects of antiserum on cell-mediated immunity in vitro (*8*) and failures in immune resistance to tumor growth in vivo deserves careful study.

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9. The HT-29 cells were supplied by J. Fogh, Sloan-Kettering Institute; and MOPC-315 cells were supplied by R. Lynch, Washington University. We thank B. Moore for technical assistance. Supported in part by PHS special research fellowship 1F03-AI53856 (W.T.S.) and by NIH grant 5R01 CA 12626.

30 July 1973

Opiate Agonists and Antagonists Discriminated by Receptor Binding in Brain

Abstract. *Receptor binding of opiate agonists and antagonists can be differentiated in vivo and in vitro. Administration of either rapidly elevates stereospecific [^{3}H]dihydromorphine binding to mouse brain extracts by 40 to 100 percent, but antagonists are 10 to 1000 times more potent than agonists; as little as 0.02 milligram of naloxone per kilogram of body weight significantly enhances opiate receptor binding. Sodium enhances antagonist binding in vitro but decreases agonist binding, a qualitative difference that may be relevant to the divergent pharmacological properties of opiate agonists and antagonists.*

Opiate antagonists are thought to occupy opiate receptor sites, preventing the access of opiates but not themselves eliciting analgesia or euphoria. Potent, long-lasting, and "pure" antagonists may provide an effective treatment for addiction, while drugs combining opiate agonist and antagonist activities offer promise as nonaddicting analgesics (*1*).

We described opiate binding in animal nervous tissue (*2*) which we attributed to specific opiate receptor sites on the basis of the stereospecificity of binding (*3*) and the close parallel between affinity for binding sites and pharmacological potency. These results have been confirmed (*4, 5*). Of 40 monkey brain regions examined, binding is most enriched in the anterior amygdala, hypothalamus, periaqueductal gray, and caudate head (*6*). Selective destruction of specific catecholamine, serotonin, and acetylcholine neural pathways in the brain does not alter binding (*6*). Receptor binding is degraded by trypsin, chymotrypsin, and phospholipase A in concentrations of less than 0.5 μg/ml and by 0.01 percent concentrations of the detergents Triton-X 100, deoxycholate, and sodium dodecyl sulfate, results suggesting that protein and lipid are important constituents of the opiate-receptor complex (*7*). The receptor is present in all vertebrate brains examined, including mammals, birds, reptiles, amphibia, and teleost fish, but cannot be detected in invertebrates such as arthropods and platyhelminths (*8*). We now report differential receptor interactions of opiate agonists and antagonists in vivo and in vitro.

Homogenates of mouse or rat brain (150 ml per gram) were incubated in triplicate in the dark for 30 minutes at 25°C in the presence of $10^{-7}M$ levorphanol or $10^{-7}M$ dextrorphan after the addition of the appropriate isotopically labeled opiate or antagonist. Samples were filtered (*2*) and washed with two 5-ml portions of tris(hydroxymethyl)-aminomethane (tris) buffer at 4°C. After extraction, the filters were counted by liquid scintillation (*2*). Specific opiate receptor activity was calculated by subtracting binding of the ^{3}H-labeled ligand in the presence of $10^{-7}M$ levorphanol from binding in the presence of $10^{-7}M$ dextrorphan, its analgeti-