the production of TTX-sensitive fast $Na^+$ channels. However, one similarity between these two studies is the effect of protein synthesis on membrane channels. There is evidence that the changes induced by exogenous mRNA are stable (*17*); that is, once the messenger has been removed, either by degradation or by changing the medium, the induced alterations persist. Alternatively, translation of the exogenous messenger taken up may lead to synthesis of stable membrane proteins with a low turnover rate, mimicking a stable genetic alteration (*18*). Although the mRNA used here is heterogeneous, one messenger must control synthesis of fast channel protein, while another could affect $P_K$, resulting in the observed increase in resting potential.

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## BCG Inhibition of Murine Leukemia: Local Suppression and Systemic Tumor Immunity Require Different Doses

Abstract. *The quantitative relationships between bacillus Calmette-Guérin (BCG) and tumor cells which are optimal for suppressing the growth of tumor cells in BCG–tumor cell mixtures are detrimental to the development of a sustained, systemic tumor rejection immunity in the LSTRA murine leukemia.*

The bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis* has become a popular agent for immunotherapy of cancer (*1*). Its action may involve combinations of several mechanisms: (i) generalized ("nonspecific") stimulation of immune responses and lymphoreticular activity, following systemic administration (not in proximity to tumor cells) (*2*); (ii) local, nonspecific destruction of tumor cells at the site of a delayed hypersensitivity response to BCG ("innocent bystander" effect) (*3, 4*); and (iii) true adjuvant effect, wherein administration of BCG in temporal and spatial proximity to tumor cells (either mixed in the same inoculum or by infiltration of tumor nodules) results in augmented development of systemic, tumor-specific rejection immunity (*5, 6*). In the treatment of human cancer, the most reliable effect of BCG has been the local destruction of tumor nodules into which the material has been injected (*7*). Systemic effects, suggestive of augmented tumor rejection immunity, have been rare, although they are the ultimate goal of immunotherapy (*8*).

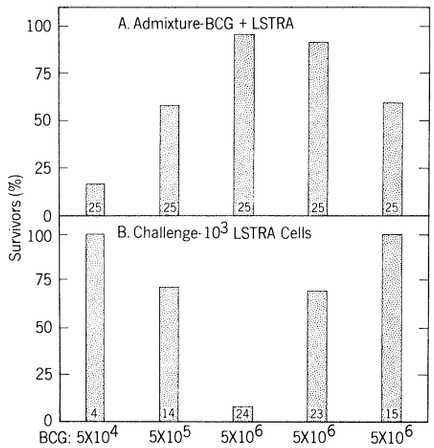


Fig. 1. Survival of mice that received intradermal injections of admixtures of LSTRA cells and BCG (pooled data of two experiments). (A) Proportion of mice in which growth of the LSTRA cells in the admixture was suppressed. (B) Proportion of mice (survivors of the admixture injections) that rejected a challenge injection of $10^3$ LSTRA cells given intradermally 42 days after injection of the admixtures (all challenge control mice died). Numbers in the bars represent the total number of mice in each group.

Little is known about the dose-response relationships of the BCG-initiated antitumor responses. In most systems diminution of BCG dose eventually eliminated the immune stimulant effects (*9*). However, under certain circumstances high BCG doses have interfered with antitumor effects (*10*), while in a chemoimmunotherapy model, BCG was effective at doses as low as 800 viable organisms (*11*). Clinical dosimetry has been largely empirical. The highest tolerated dose is sought, and tends to be limited more by the concentration of the vaccines available than by biologic considerations. We report here that in one animal model the doses of BCG and tumor cells which regularly produced local suppression of tumor growth did not induce detectable tumor immunity, while the doses that were required to induce tumor immunity were relatively ineffective in achieving local suppression of tumor growth.

In this study, local tumor suppression was evaluated by the growth or suppression of tumor cells mixed with BCG before injection (*3*); adjuvant effects were assessed by the resistance to tumor challenge in mice which had suppressed the original tumor-BCG inoculum (*5*). The LSTRA Moloney virus–induced leukemia (*12*), of BALB/c origin, was studied in male $(BALB/c \times DBA/2)F_1$ hybrids (*12*). The tumor was maintained by weekly intraperitoneal passage of ascites tumor (0.2 ml of a 1 : 1000 dilution of ascites fluid in minimum essential medium, usually containing $2 \times 10^4$ to $4 \times 10^4$ viable tumor cells). Intradermal inoculation of $10^3$ cells results in greater than 95 percent mortality, and

Table 1. Survival of mice after intradermal injection of BCG and LSTRA cells, either at separate sites or mixed before injection, and after subsequent intradermal injection (challenge) of $10^3$ LSTRA cells into mice that had survived the first injection. Fractions give the number of survivors (S) in a group divided by the number of animals ($N$) which received the injection; median death refers to the number of days after injection by which $\geq$ 50 percent of mortalities had occurred.

| Dose | | | Injection | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | BCG + LSTRA (day 0) | | Challenge (day 42) | | BCG + LSTRA (day 0) | | Challenge (day 42) | |
| Ratio | BCG | LSTRA | | S/$N$ | Median death (days) | S/$N$ | Median death (days) | S/$N$ | Median death (days) | S/$N$ | Median death (days) |
| 50 : 1 | $5 \times 10^4$ | $10^3$ | Separate | 1/10 | 14 | 1/1 | | 0/15 | 15 | | |
| | | | Mixed | 4/10 | 15 | 4/4 | | 0/15 | 16 | | |
| 500 : 1 | $5 \times 10^5$ | $10^3$ | Separate | 0/10 | 15 | | | 1/15 | 15 | 0/1 | 31 |
| | | | Mixed | 7/10 | 29 | 6/7 | 15 | 7/14 | 24 | 4/7 | 12 |
| 5000 : 1 | $5 \times 10^6$ | $10^3$ | Separate | 0/10 | 14 | | | 0/15 | 15 | | |
| | | | Mixed | 9/10 | 19 | 2/9 | 14 | 15/15 | | 0/15 | 13 |
| 500 : 1 | $5 \times 10^6$ | $10^4$ | Separate | 0/10 | 13 | | | 0/15 | 12 | | |
| | | | Mixed | 9/10 | 17 | 4/9 | 14 | 14/15 | 16 | 12/14 | 11 |
| 50 : 1 | $5 \times 10^6$ | $10^5$ | Separate | 1/9 | 11 | | | 0/15 | 10 | | |
| | | | Mixed | 8/10 | 39 | 8/8 | | 7/15 | 13 | 7/7 | |
| Challenge controls | | | | | | 0/5 | 13 | | | 0/5 | 12 |

$10^2$ cells produce 50 to 70 percent mortality. Phipps strain BCG (*12*) was stored at −70°C. For use in experiments it was thawed rapidly and diluted in 7H9 Middlebrook broth. Groups of 10 or 15 mice received intradermal injections (0.05 ml) of BCG-LSTRA cell mixtures (Table 1); controls received similar numbers of cells and organisms at separate (contralateral) sites. More than 97 percent of controls died of progressive tumor growth. In mice that received mixed inocula containing a constant number of LSTRA cells, the frequency of suppression of tumor growth was directly related to the dose of BCG organisms in the mixture. When the BCG dose was constant, the frequency of tumor suppression in the admixtures was inversely related to the dose of LSTRA cells. Within the limited dose ranges tested, the efficiency of local tumor suppression was directly related to the BCG : LSTRA cell ratio.

Forty-two days after the initial inoculation, tumor-free survivors and age-matched, untreated controls were challenged with intradermal injection of $10^3$ LSTRA cells (0.05 ml) in the ventral skin. All challenge controls died of the tumor inoculum. Mice listed as survivors have remained tumor-free for more than 120 days in the first experiment and more than 60 days in the second experiment. There was no evidence of regrowth of the first inoculum following challenge. Among survivors that had received equal numbers of tumor cells ($10^3$) in the initial inoculum, resistance to challenge was best if the admixture had contained few BCG ($5 \times 10^4$), and was rare if the admixture had contained a larger dose of BCG ($5 \times 10^6$). Among the surviving mice that had received equal numbers of BCG ($5 \times 10^6$) in the initial inoculum, resistance to challenge was rare if the admixture had contained few ($10^3$) tumor cells and was common if the admixture had contained a larger tumor cell dose ($10^5$ cells). Overall, the proportion of immune mice was high (100 percent) if the BCG : LSTRA ratio was low (50 : 1), and the proportion of immune mice was low (8 percent) if the BCG : LSTRA ratio was high (5000 : 1).

The results of the two experiments have been pooled and summarized in Fig. 1. Conditions ideal for tumor suppression were inadequate for induction of tumor immunity; optimal immunity developed when conditions were at the threshold for tumor suppression. Local suppression of tumor cells was favored by a high BCG : LSTRA cell ratio, while immunization was favored at lower ratios. These data reflect only five points in a larger potential dose-ratio matrix, so it is not possible at this time to decide whether the BCG : LSTRA cell ratio was the critical variable. In the present experiments, the absolute doses of LSTRA or BCG did not seem to be the limiting factors for immunization against tumor challenge, since the lowest tumor cell dose was immunogenic with appropriate BCG doses, and the highest BCG dose was not inhibitory for tumor immunity when adequate LSTRA cells were present. This is the first description of reciprocal dose-response relationships for two different types of BCG-initiated tumor inhibition.

These observations are similar to those reported for a different murine tumor system, with *Corynebacterium parvum* as the immune stimulant (*13*). Scott found that the best immunity to tumor cell challenge was obtained with the lowest dose of *C. parvum* tested (3.5 μg) and with the highest dose of irradiated tumor cells used ($10^7$). In that study it was not possible to evaluate the effect of the tumor cell–stimulant dose ratio, nor could the efficacy for immunization be compared to the efficacy for local suppression.

We have referred to the role of BCG in the induction of tumor immunity as an "adjuvant effect," but it could be working here by other, nonadjuvant means. The immunity detected could be due to unaugmented immunogenicity of the tumor cells in the initial "vaccine" inoculum. Presence of BCG in the vaccine may simply be an effective means of preventing progressive growth of the immunizing tumor cells in the vaccine, comparable to the use of subthreshold cell doses and the ligation or excision of solid tumors for immunization (*14*). In our experience, mice that survive subthreshold inocula of LSTRA, or that survive inocula of $10^4$ LSTRA cells because of chemotherapy (with or without BCG), are usually unable to reject a challenge injection of $10^3$ LSTRA cells. Animals are not cured by excision of tumors at the site of LSTRA injection if the surgery is later than 5 days after injection. In other animal tumor models, irradiation of tumor cells can prevent growth of immunizing vaccines without destroying immunogenicity (*5, 13, 15*). That approach may be useful in this system.

We have assumed that the resistance to

challenge in these experiments was due to tumor rejection immunity, but passive transfer and specificity experiments would be required to establish that as fact. The resistance we have demonstrated may not be very strong, even in those cohorts which were 100 percent resistant to the challenge with $10^3$ cells, but this is the first report of such a high frequency of resistance to LSTRA cell challenge induced by any prior treatment with LSTRA cells.

We do not suggest that these data can be extrapolated directly to every clinical situation. These observations may apply to immunization with tumor cell–adjuvant mixtures, but they may not be of general relevance to the development of immunity following intratumor injection of immune stimulants. The results need confirmation in other laboratories and ought to be extended in this and other animal tumor models. This study has illustrated the potential complexities in the design of rational immunotherapy protocols: When administering immune stimulants, more may not be better; regression of injected lesions may not correlate with systemic immunization against tumor rejection antigens.

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## Melatonin Inhibition of the Neonatal Pituitary Response to Luteinizing Hormone–Releasing Factor

Abstract. *Neonatal rat anterior pituitary glands treated in organ culture with 1 nanomolar luteinizing hormone–releasing factor (LRF) showed a tenfold increase in medium luteinizing hormone (LH) concentrations over control values. Simultaneous treatment of the glands with 1 nanomolar melatonin significantly reduced the stimulatory effect of LRF on release of LH. This finding indicates that melatonin can act directly on the neonatal pituitary to inhibit the LH response to LRF.*

Numerous studies (*1*) have shown that the antigonadotropic effects of the pineal gland may be mediated in part by melatonin. However, the precise mechanism by which melatonin inhibits reproductive function remains to be resolved. We now report that melatonin can act directly on the pituitary gland to suppress the release of luteinizing hormone (LH) induced by luteinizing hormone–releasing factor (LRF).

The effect of melatonin on the LH response to synthetic LRF (*2*) was studied in organ culture with the use of anterior pituitary glands from 5-day-old female Sprague-Dawley rats. The method of culture used in this study (*3*) is identical to that developed in our laboratory for the culture of pineal glands (*4*). With this system, there is no apparent cellular necrosis in 5-day-old pituitaries incubated for 4 days (*3*).

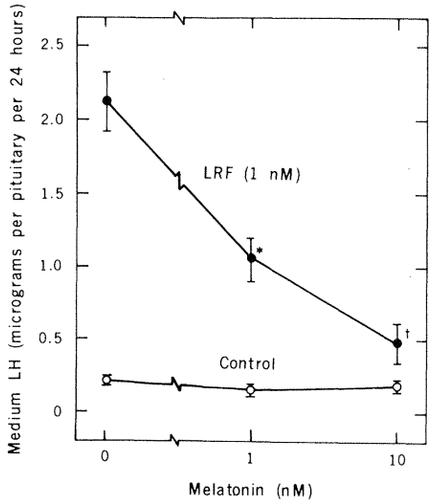


Fig. 1. Effect of melatonin on LRF-induced release of LH. After 24 hours of culture under control conditions, anterior pituitary glands from 5-day-old female rats were treated for 24 hours with control medium, melatonin (1 or 10 n*M*), LRF (1 n*M*), or LRF (1 n*M*) plus melatonin (1 or 10 n*M*). Each point represents the mean response of four anterior pituitaries. Vertical lines represent standard errors. *Significantly less than 1 n*M* LRF and 0 melatonin value; $P < .01$. †Significantly less than 1 n*M* LRF and 0 melatonin value; $P < .001$.

After 24 hours of culture under control conditions, pituitary glands were transferred to fresh medium containing the test substances. Half of the glands were stimulated with LRF (1 n*M*) either alone or in the presence of melatonin (1 or 10 n*M*). The remaining half of the glands served as controls and were incubated either in control medium or in medium containing only melatonin (1 or 10 n*M*). The culture was terminated after a 24-hour treatment period. The LH content of the medium was measured by double antibody radioimmunoassay, in which materials supplied by the Rat Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases were used. Values are expressed in terms of the reference preparation NIAMDD-Rat-LH-RP-1. Melatonin in culture medium at a concentration of 1 $\mu M$ does not interfere with the measurement of LH in our assay (*5*). Statistical analysis was made by Student's *t*-test.

As shown in Fig. 1, LRF treatment in the absence of melatonin produced a tenfold increase in the concentrations of LH in the medium over control values. However, simultaneous treatment of the pituitary glands with LRF and 1 n*M* melatonin resulted in a highly significant ($P < .01$) reduction of LH secretion. At 10 n*M*, melatonin suppressed the LRF-induced release of LH to 14 percent of the response obtained with LRF alone. Melatonin had no detectable effect on control concentrations of LH at either of the doses used.

These results indicate that melatonin can act at the pituitary level to suppress