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Conditioning: A New Approach to Immunotherapy[1]

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ABSTRACT

It has been demonstrated by Parmiani *et al.* (Int. J. Cancer, *29:* 323–332, 1982) that a significant protective effect can be obtained against the transplanted syngeneic YC8 lymphoma by prior immunization of BALB/c mice with normal allogeneic DBA/2 spleen cells. Using this well established tumor model, we investigated a novel approach, conditioning of specific immunotherapeutic activity. For this purpose, we used the odor of camphor as the conditioning stimulus and allogeneic DBA/2 spleen cells as unconditioning stimulus. We associated the conditioning and unconditioning stimuli two, three, and four times. Following this the conditioned animals were reexposed to the odor of camphor only. In each case, we observed a delay in tumor growth and in some instances the conditioned group performed better than the immunotherapy control group. These results indicate that a limited number of treatments with the antigen is better than the continuous treatment in maintaining the immunity and the homeostasis of the system.

INTRODUCTION

In previous studies, we have demonstrated that mice conditioned to camphor and poly(I:C)[3] injections could resist MOPC 104E myeloma growth *in vivo* when the CND mice were exposed to the odor of camphor only. Similarly conditioned mice when given injections of myeloma cells did not resist tumor growth in the absence of subsequent exposure to camphor odor. Camphor odor itself had no therapeutic benefit (1). Of particular importance is that the NK cell response can similarly be conditioned with camphor odor and poly(I:C) injections (2). These results support the view that resistance to cancer can be conditioned under the proper settings. These studies would be more relevant clinically if conditioning can be achieved in an animal in which tumor is already present. The studies of Parmiani *et al.* (3) showed that significant protective effect can be obtained against the outgrowth of the transplanted syngeneic YC8 lymphoma by prior immunization of the host (BALB/c mice) with normal allogeneic (DBA/2) spleen cells. *In vivo* experiments with the Winn assay and with nude mice indicated that the resistance induced by alloimmunization was mediated by T-cells. The inability of BALB/c-*nu*/*nu* mice to develop resistance upon alloimmunization was taken as evidence that host T-cells were important and NK cells were not involved, since nude mice have an efficient NK cell activity. We investigated the possible application of conditioning specific immunotherapy in the YC8 tumor model system. These studies were aimed at answering three important questions: (*a*) How soon can we discern the effects of conditioning? (*b*) How reproducible is the conditioning effect? (*c*) Is the effect of conditioning more powerful than immunotherapy?

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from Charles River Laboratories (Wilmington, DE) and maintained on standard laboratory chow and water *ad libitum*. Mice were used when 8–12 weeks old.

Immunizations. BALB/c mice (*H-2*^{d}) receiving DBA/2-*H-2*^{d} spleen cell immunizations were given 1.5×10^7 DBA/2 spleen cells/mouse i.p. in 0.1 ml of 0.9% NaCl solution (saline). Immunizations were initiated 5 days after tumor implantation with 5×10^4 YC8 cells s.c.

YC8 T-Cell Lymphoma Model. YC8 is a Moloney virus induced T-cell lymphoma which is *H-2*^{d}, Thy-1.2$^+$ (3). The tumor is maintained *in vitro* and/or *in vivo* in BALB/c mice. The YC8 cell line was a generous gift from Dr. M. P. Colombo, Wistar Institute.

Tumor Measurement. Tumor growth s.c. was measured and recorded as the mean diameter of two measurements at 90 degrees. The results were expressed as the mean tumor size in mm ± SEM.

Conditioning the Immunotherapeutic Response with Camphor Odor. Using the YC8 tumor model, we studied the effects of conditioning the immunotherapeutic response. This was done by associating the camphor odor (CS) with the immunization (US). In the first study, 6 groups of animals were used (Table 1). There were 10 mice in each group; all animals were given injections of 5×10^4 YC8 cells s.c. Conditioning trials were done in which two and three associations were made between camphor exposure and immunization (CS/US). CND2 denotes a group receiving two CS/US associations on days 5 and 7 followed by camphor only on days 10, 12, 19, 26, 33, and 40. CND3 denotes a group receiving three CS/US associations on days 5, 7, and 10 followed by camphor alone on days 12, 19, 26, 33, and 40. We also included conditioned zero (CNDo) groups for both two and three associations. The CNDo groups were conditioned in the same way as the CND groups but differed in that subsequently the animals were not exposed to the odor of camphor. An untreated control group and an immunotherapy group were also included. The immunotherapy group received immunizations on days 5, 7, 10, 12, 19, 26, 33, and 40. Five days after tumor implantation, the CND2, CNDo2, CND3, and CNDo3 groups were exposed to camphor odor for 1 h. This was done by placing animal cages into a cabinet and placing a small bottle containing camphor dissolved in mineral oil on top of each cage top. The camphor in mineral oil was warmed prior to placing on the cages, and a second cage was inverted on top of each cage to contain the camphor vapors. The cabinet was closed and the animals were exposed for 1 h. Immediately after exposure to camphor each animal in the CND and CNDo groups were given i.p. injections of 0.1 ml of 1.5×10^7 DBA/2 spleen cells. The schedule of exposure to camphor odor and immunization is shown in Table 1. The immunotherapy group received DBA/2 spleen cells only on the days indicated. The control group was treated with saline on the days indicated.

In the second series four conditioned associations were made. In this study 4 groups of mice, CND, CNDo, saline control, and immunotherapy groups with 10 mice each, were used. The protocol used in the treatment is shown in Table 2. Mice in all four groups were given s.c. injections of 5×10^4 YC8 cells on day 0. On day 5, the CND and CNDo groups were exposed to camphor odor for 1 h. Immediately after exposure to camphor, each animal in the CND and CNDo group was given an i.p. injection of 0.1 ml of 1.5×10^7 DBA/2 spleen cells. The exposure to camphor odor and immunization were given on days 5, 7, 10, and 12. The CND group was subsequently exposed to camphor odor only on days 19, 26, 33, and 40. The CNDo group received no further exposure to camphor odor and served as a control for the CND

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[3] The abbreviations used are: poly (I:C), polyionosonic:polycytidilic acid; CND, conditioned; CS, conditioning stimulus, camphor; US, unconditioning stimulus, DBA/2 spleen cells; NK, natural killer cells; CNDo, conditioned mice not reexposed to CS; CND2, CND3, CND4, CS/US associations were made two, three, and four times, respectively; MST, median survival time.

Table 1 *Treatment schedule for the conditioning of the CND2 and CND3 groups*

	Treatment days							
Group[a]	5	7	10	12	19	26	33	40
CND2[b]	CD	CD	C	C	C	C	C	C
CNDo2	CD	CD						
CND3	CD	CD	CD	C	C	C	C	C
CNDo3	CD	CD	CD					
IMM[c]	D	D	D	D	D	D	D	D
Saline	S	S	S	S	S	S	S	S

[a] Each group contained 10 mice. All mice were given s.c. injections on day 0 of 5×10^4 YC8 tumor cells.
[b] Mice in the CND and CNDo groups were exposed to odor of camphor (C) for 1 h followed by an i.p. immunization of 1.5×10^7 DBA/2 spleen cells (D) within 15 min after exposure to C.
[c] Standard immunotherapy (IMM) group received only immunizations with D on the designated days.

Table 2 *Protocol for conditioning mice using four CS/US associations*[a]

	Treatment days							
Group[a]	5	7	10	12	19	26	33	40
CND4[b]	CD	CD	CD	CD	C	C	C	C
CNDo4	CD	CD	CD	CD				
Saline	S	S	S	S	S	S	S	S
IMM[c]	D	D	D	D	D	D	D	D

[a] Each group contained 10 mice. All mice were given s.c. injections on day 0 of 5×10^4 YC8 tumor cells.
[b] Mice in the CND and CNDo groups were exposed to odor of camphor (C) for 1 h followed by an i.p. immunization of 1.5×10^7 DBA/2 spleen cells (D) within 15 min after exposure to C.
[c] Standard immunotherapy (IMM) group received only immunizations with D on the designated days.

group. The saline control group was treated with saline on the indicated days and weekly thereafter. The immunotherapy group received DBA/2 spleen cells only, on the indicated days and weekly thereafter. All animals were inspected daily and monitored for tumor growth by caliper measurements and for survival.

RESULTS

Conditioned Immunotherapy. In this study mice were given injections with YC8 tumor cells s.c. on day 0 and either two or three CS/US associations were made starting from day 5. When animals were conditioned twice on days 5 and 7 and exposed to camphor only on days 10, 12, 19, 26, 33, and 40, some effect was noticed in the CND2 group over the CNDo2 group. The CNDo2 group was conditioned the same way as the CND2 but not exposed to camphor thereafter. The CND2 group showed a decrease in tumor size and a delay in growth between 12 and 19 days (7-day delay) before the average tumor size progressed (Fig. 1). Tumor size measurements were analyzed for significance by regression analysis (CND *versus* CNDo, $P = 0.798$). Interestingly, the response in the CND2 group was better than in the immunotherapy group; however, there was no statistically significant difference (CND *versus* immunotherapy, $P = 0.7627$). The untreated control group showed the greatest rate of tumor growth. The MSTs of CND2, CNDo2 and the saline control groups were 36, 34, and 26 days, respectively (Fig. 2). The saline group showed the least resistance as they were not immunized. The immunotherapy group had a MST of 40 days. Interestingly, the CND2 group had a 10% cure rate (1 of 10). There were no cures in any of the other groups. The survival benefit was not statistically significant when analyzed by generalized Wilcoxon test (CND *versus* CNDo, $P = 0.127$, CND *versus* immunotherapy, $P = 0.651$).

When animals were conditioned 3 times on days 5, 7, and 10 and exposed to camphor only on days 12, 19, 26, 33, and 40 a greater effect was noticed in the CND3 group over the CNDo3 group. The CNDo3 was conditioned in the same way as the CND3 group but was not exposed to camphor thereafter. The CND3 group showed a delay in growth of tumor between 12 to 24 days (12-day delay, Fig. 3). Tumor growth rate was analyzed by regression analysis for significance; where CND *versus* CNDo, $P = 0.0004$ and CND *versus* immunotherapy, $P = 0.0028$. What again appears remarkable is the animals in the CND3 group showed an earlier delay in the growth of tumor and a better response than the immunotherapy group. These results suggest that the central nervous system can be conditioned to assist in the immune regulation to the tumor *in vivo*. Interestingly, the growth of tumor in the CNDo3 group paralleled the immunotherapy group. Fig. 4 shows the survival data for this experiment. In the CND3 group the MST was 45 days but 50% of the animals in this group lived as long as 52 days. In comparison the MST was only 32 days in the CNDo3 group and 50% of the animals survived as long as 35 days. This disparity seems remarkable considering that the CNDo3 group

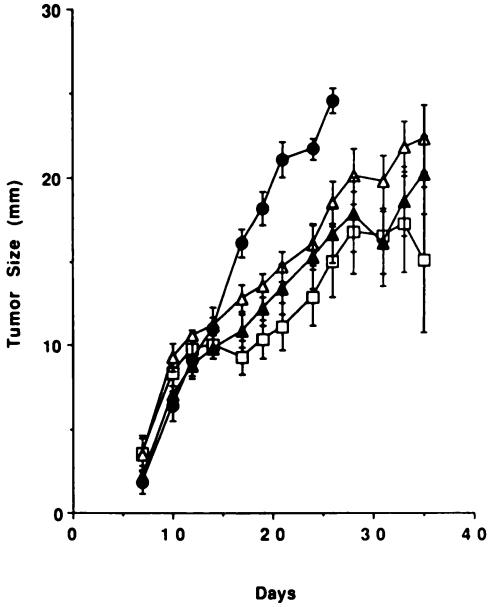

Fig. 1. Effect of two CS/US associations on the rate of growth of YC8 lymphoma *in vivo*. Mice were given injections of 5×10^4 tumor cells s.c. on day 0 and conditioning was done on days 5 and 7. CND2, conditioned group (□); CNDo2, conditioned but not subsequently reexposed to camphor odor (△); control, treated with saline (●); immunotherapy, injected with DBA/2 spleen cells (▲). Statistical comparison was performed by regression analysis; CND *versus* CNDo, $P = 0.798$; CND *versus* control, $P = 0.0047$, and CND *versus* immunotherapy, $P = 0.7627$.

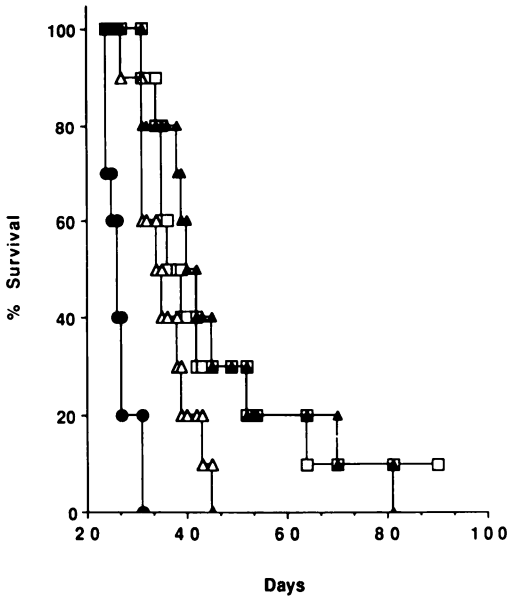

Fig. 2. Effect of conditioning two times on survival of mice bearing the YC8 lymphoma. The definition of the symbols and the groups are same as Fig. 1. The generalized Wilcoxon test was used to determine the statistical significance of the groups; CND *versus* CNDo, $P = 0.127$; CND *versus* control, $P = 0.00009$; and CND *versus* immunotherapy, $P = 0.651$.

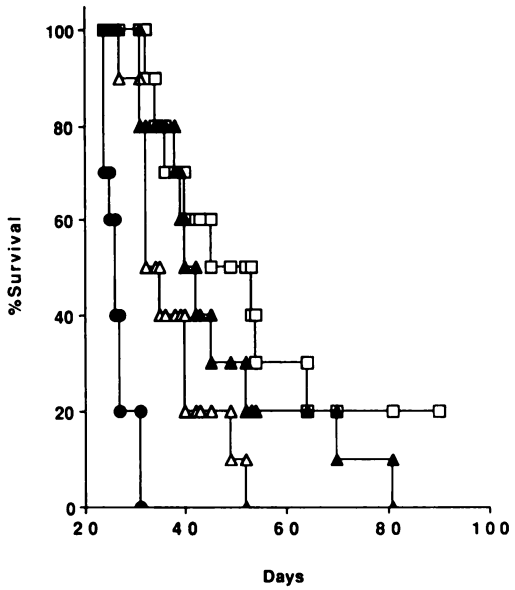

Fig. 4. Effect of conditioning three times on survival of mice bearing the YC8 lymphoma. The groups and definition of the *symbols* are same as in the legend for Fig. 3. CND *versus* CNDo, $P = 0.022$; CND *versus* control, $P = 0.0004$; and CND *versus* immunotherapy, $P = 0.386$.

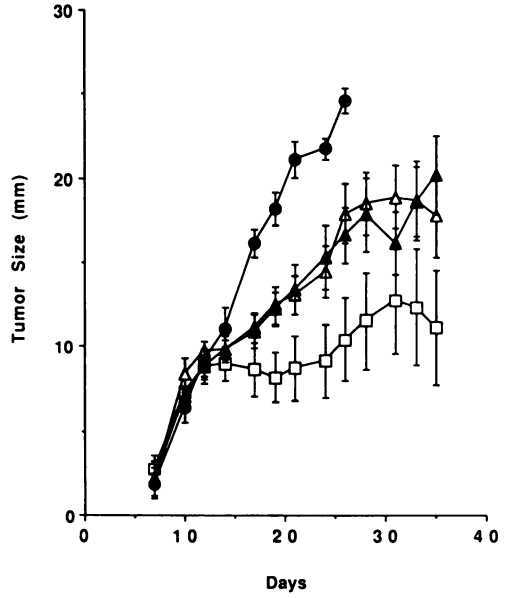

Fig. 3. Effect of three CS/US associations on the rate of growth of YC8 lymphoma *in vivo*. Mice were given injections of 5×10^4 cells s.c. on day 0 and conditioning was done on days 5, 7, and 10. CND3, conditioned group (□); CNDo3, conditioned but not subsequently reexposed to camphor odor (△); control, treated with saline (●); immunotherapy, injected with DBA/2 spleen cells (▲). CND *versus* CNDo, $P = 0.0004$; CND *versus* control, $P = 0.0001$; and CND *versus* immunotherapy, $P = 0.0028$.

was conditioned in the same fashion as CND3, the only difference being that the CNDo3 group was not allowed to smell the odor of camphor. The saline and immunotherapy groups had a MST of 26 and 40 days, respectively. Survival curves were compared for significance by the generalized Wilcoxon test. *P* for CND *versus* CNDo was 0.022 and that for CND *versus* immunotherapy was 0.386. Again, cures were recorded in the CND3 group, (2 of 10 animals; 20% regressed their tumors). There were no cures in the CNDo3 group. Possible additional evidence that camphor alone mimics immunization is shown by the following example. As measured by tumor size, mice immunized 3 times generally show a slower growth of their tumor than mice immunized twice. For example, CNDo3 (3 times immunized, Fig. 3) actually showed slower growth of their tumor than CNDo2 (2 times immunized, Fig. 1). This is expected because 3 immunizations impart slightly more resistance than 2 immunizations. It should be noted that the CND2 group (2 times conditioned) show even smaller tumors than CNDo3 (3 times immunized). This suggests that animals in CND2 act as if they were being immunized more than 2 times by exposure to camphor odor.

Two separate studies were done to assess the effects of four CS/US associations on tumor growth. In this study mice were initially given injections of YC8 s.c. on day 0. The neoplasm was allowed to establish itself and on day 5 animals were conditioned to camphor odor and DBA/2 spleen (antigen) injections for four conditioning periods on days 5, 7, 10, and 12. Weekly thereafter the CND group was exposed to camphor odor only (Table 2). Data from one of the studies are given. The rate of growth of YC8 in the mice conditioned to camphor and DBA/2 spleen cell injections is shown (Fig. 5). Weekly reexposure to camphor odor appears to have imparted greater resistance. The CNDo group was conditioned in identical manner along with the CND group but was subsequently not reexposed to the odor of camphor. A camphor control group was exposed to the odor of camphor on days 5, 7, 10, and 12 and given saline injections instead of DBA/2 spleen cells. This group was subsequently reexposed to camphor odor weekly thereafter.

Fig. 5 shows the rate of growth of YC8 in mice conditioned with camphor odor and immunization with DBA/2 spleen cells. There was a suppressive effect on the growth of YC8 tumor in the mice which were subsequently exposed to the odor of camphor (CND *versus* camphor control, $P = 0.0125$; CND *versus* CNDo, $P = 0.0039$). The tumor size measurements of the second experiment (data not given) was also analyzed for statistical significance where *P* values for CND *versus* CNDo and CND *versus* control were 0.0614 and 0.0159, respectively. Fig. 6 shows the survival rate and cures for each group. The CND group had the greatest MST (41 days). The MST for

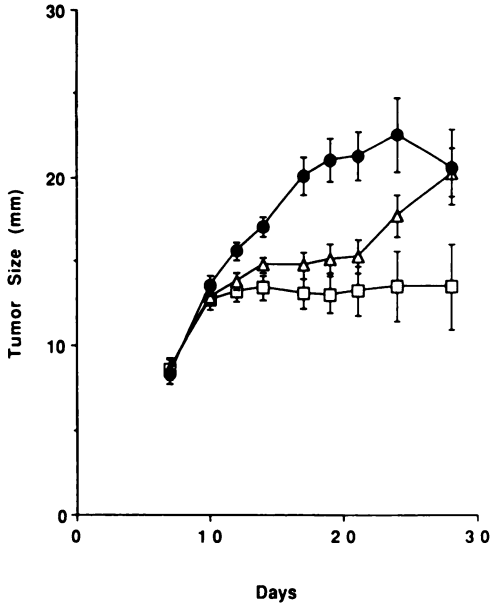

Fig. 5. Effect of conditioning (four CS/US associations) on the rate of growth of YC8 lymphoma *in vivo*. Mice were given injections of 5×10^4 YC8 cells s.c. Conditioning was initiated 5 days after tumor implantation. CND, conditioned group, subsequently reexposed to camphor odor only (□); CNDo, conditioned but not reexposed to camphor odor (△); camphor control, exposed to camphor odor only (●). *P* values were obtained by regression analysis. CND *versus* CNDo, $P = 0.0039$; and CND *versus* control, $P = 0.0125$.

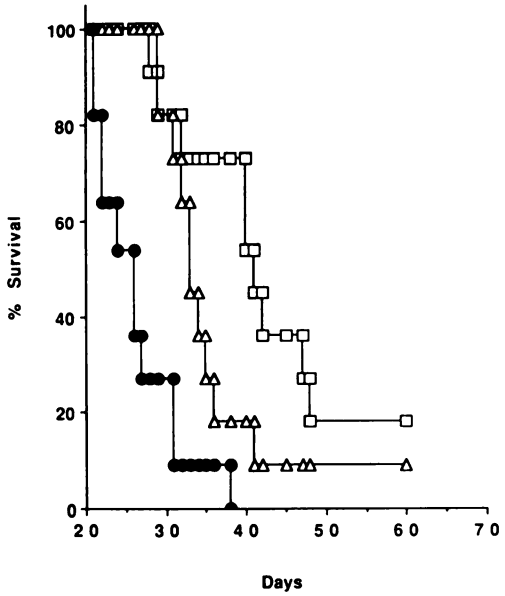

Fig. 6. Effect of conditioning on the survival of mice bearing the YC8 lymphoma. The groups and definition of the *symbols* are given in the legend for Fig. 5. A generalized Wilcoxon test was used to determine the statistical significance of the groups; CND *versus* CNDo, $P = 0.132$; and CND *versus* control, $P = 0.00033$.

CNDo group was 33 and the camphor control was 26 days. The survival data were compared by the generalized Wilcoxon test, CND *versus* camphor control, $P = 0.00033$; CND *versus* CNDo, $P = 0.132$. Comparison of the survival curves of the second experiment (data not shown) demonstrated borderline significance for CND *versus* CNDo ($P = 0.059$) and significance for CND *versus* control ($P = 0.00003$). These results substantiate the observed data of the first experiment. We saw improvement in the CND group in retardation of tumor growth and increase in survival. There were a number of regressors (CND, 2 of 11; CNDo, 1 of 11). There were no survivors (0 of 11) in the camphor control group. In the second experiment again there were a few regressors (CND, 2 of 10; CNDo, 1 of 10; immunotherapy, 1 of 10, and control, 0 of 10).

Since two similar experiments were performed to establish the reliability and reproducibility of the observations, the data from both of the experiments were combined and analyzed for statistical significance (4, 5). First, the data were analyzed for difference in experiments by the rank regression procedure and the generalized Wilcoxon test. The combined data of the two experiments of each group were adjusted for the difference in experiments and analyzed for significance. The results observed were significant for overall tumor growth rates (CND4× *versus* CNDo4×, $P = 0.0055$) and survival curves (CND4× *versus* CNDo4×, $P = 0.017$).

DISCUSSION

In animals with an ongoing disease process, the application of Pavlovian conditioning to alter or retard the process is clearly in the realm of possibility. Pairing the odor of camphor with injections of poly(I:C) has been shown to condition the NK cell response (2). Ghanta *et al.* (1) have shown that this conditioning paradigm can be used to retard and/or possibly regress the growth of MOPC 104E plasmacytoma. In a few animals, 10–20%, total reversal of the tumor growth and cancer free survival of more than 100 days were reported. The mechanism in this instance was not clear since the MOPC 104E tumor itself is not NK sensitive. Thus, it is likely that other antitumor activity may have been conditioned by pairing camphor odor with poly(I:C) injection. Macrophages may be induced to control the proliferation of myeloma by cytostatic processes has been suggested (6).

Other conditioning paradigms have been used to alter disease processes. Ader and Cohen (7, 8) conditioned immunosuppression using saccharin as the CS and cyclophosphamide as the US. They were able to condition the immunosuppression of a spontaneously developing autoimmune disorder, systemic lupus erythematosus in the female NZB × NZW F_1 mouse, resulting in an improvement in survival. Klosterhalfen and Klosterhalfen (9) using a similar paradigm were able to condition the immunosuppression of bacterial adjuvant induced arthritis in rats.

Gorczynski *et al.* (10) conditioned the immunosuppression in BALB/c mice to MOPC 315 plasmacytoma. In such conditioned animals the exposure to saccharin allowed the myeloma to grow at a faster rate. Cimetidine which binds histamine type II receptor on suppressor T-cells (11) abrogated the conditioned immunosuppressive response.

The specificity of these conditioned responses to alter the disease processes have not been clearly established as in the YC8 immunotherapy model. We have adopted an immunotherapy model in which the host's own immune system is activated against a growing syngeneic tumor. This model more accurately reflects the real life situation, where tumor cells are already present before any immunotherapy treatment can be instituted. We have utilized camphor odor in combination with active immunotherapy. Camphor has no tumoricidal activity against the YC8 tumor and cannot ablate tumor induced suppressor cells in the host. Therefore the therapeutic effect on the YC8 tumor observed in mice exposed to camphor coupled with active immunotherapy appears to be the result of camphor mimicking active immunostimulation producing resistance to established neoplasms *in vivo*. These studies demonstrate the bidirectionality between the CNS and immune system and provide evidence for the first time that a specific immune

response can be focused through the CNS to bring about resistance to and regression of a growing neoplasm *in vivo*. More importantly, these studies support the view that regulation of tumor growth (as measured by growth delay) through conditioning was more effective than our best immunotherapy treatment regimen which was arrived at empirically.

The results of effect of the number of CS/US associations on tumor growth and survival clearly demonstrate that three and four associations were better than two associations in regulating tumor growth rate (CND *versus* CNDo of two, three, and four times, $P = 0.798$, 0.0004, and 0.0055, respectively). Similarly, three and four associations have better therapeutic effect in prolonging the median survival and producing a small number of cures. Comparison of survival curves with the generalized Wilcoxon test demonstrated the therapeutic benefit of the three and four associations over two associations (CND *versus* CNDo of two, three, and four times, $P = 0.127$, 0.022, and 0.017, respectively). The results also demonstrate that three associations might be optimal and superior to four associations.

These results taken together show: (*a*) the effect of conditioning can be discerned after two CS/US associations; (*b*) the effect produced by conditioning is reproducible; and (*c*) the immunotherapeutic response in conditioned mice appears to be better than that produced by the standard immunotherapy regimen which we developed.

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