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Conditioning of the Secondary Cytotoxic T-Lymphocyte Response to YC8 Tumor

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GHANTA, V. K., N. S. HIRAMOTO, S.-J. SOONG AND R. N. HIRAMOTO. *Conditioning of the secondary cytotoxic T-lymphocyte response to YC8 tumor.* PHARMACOL BIOCHEM BEHAV **50**(3) 399–403, 1995.—Studies from our laboratory demonstrated that conditioned resistance to the syngeneic YC8 lymphoma was established by multiple conditioned stimulus (CS)/unconditioned stimulus (US) associations. The conditioned stimulus used was exposure to the odor of camphor for 1 h and the unconditioned stimulus was an injection of DBA/2 spleen cell alloantigen that shares minor histocompatibility determinants with the YC8 lymphoma. To demonstrate a cellular basis for immune resistance to the YC8 tumor, BALB/c mice primed with DBA/2 spleen cell alloantigen were conditioned using a single trial CS/US association paradigm. Conditioned animals showed a measurable conditioned elevation of the cytotoxic T-lymphocyte (CTL) response to the YC8 tumor. Control groups in which the CS and US were not given in the proper sequence were unable to mount a conditioned response. These studies show that a secondary CTL response can be upregulated by the central nervous system (CNS).

Conditioning CTL actvity Secondary CTL response YC8 lymphoma Anticancer response Immunotherapy

CONDITIONING has been used in an effort to modulate resistance to cancer (6,7,10). The use of conditioning as an additional modality has been largely ignored and neglected as being too weak to produce measurable responses. We used the YC8 lymphoma model developed by Parmiani et al. (17) for these studies because a normal tissue vaccine is available for immunization against the YC8 tumor. Significant protection can be obtained against transplanted syngeneic YC8 tumor cells by prior immunization of BALB/c mice with normal allogeneic DBA/2 spleen cells. The DBA/2 spleen cells used as the alloantigen are known to share minor histocompatibility determinants with YC8 tumor. The protection acquired by immunization can be passively transferred by immune lymphocytes, and in studies using adoptive chemoimmunotherapy (ACIT), regression of established tumor growth was observed (10).

We conditioned the specific immunotherapeutic resistance in BALB/c mice by using camphor odor as the conditioned stimulus and allogeneic DBA/2 spleen cells as the unconditioned stimulus. The CS/US stimuli were paired two, three, and four times, and following this period of training the animals were reexposed to the odor of camphor only. In each instance we observed a delay in tumor growth in the conditioned animals (6). The effect produced by ACIT was also conditioned, and in conditioned animals a greater incidence of regressions and cures was observed (7). The basis for this conditioned resistance has not been defined. Here we report the conditioning of the syngeneic, secondary cytotoxic T-lymphocyte response against the YC8 lymphoma and demonstrate that the conditioned resistance is mediated by signals from the CNS.

METHOD

Mice

Six-week-old female BALB/c mice (H-2^d) were purchased from Charles River Breeding Laboratories (Wilmington, DE) and maintained on standard rodent chow and water ad lib with 12L : 12D cycle (lights on at 0600 and off at 1800 h). The animals were allowed to adapt to our vivarium surroundings for at least 1 week before use in the experiments.

Conditioning CTL Response

All conditioning procedures (association, reexposure) were started between 0700 and 0730 h. The exposure to the odor stimulus and treatment with US were completed by 0830 h. A

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1 oz. block of camphor (C) was partially dissolved in mineral oil (one block to about 150 ml of mineral oil) while stirring on low heat. Camphor exposure was carried out inside a cabinet in a different laboratory. The camphor/mineral oil mixture (30 ml) in a small glass container was heated in a microwave oven for 1 min, and then placed upon the cage top. Another empty cage was inverted over the cage holding the animals to contain the camphor odor. This was done inside the cabinet away from where the other animals were housed, and care was taken to prevent the camphor odor from reaching the control animals. Mice were exposed to camphor odor in this way for 1 h.

The mice of each group were housed separately in individual cages for 1 week prior to the performance of the experiment. On day 0, the CND and CNDo groups were moved to the room with the cabinet and exposed to camphor odor for 1 h without food and water. After exposure to camphor odor, the animals were injected with 1.5×10^7 DBA/2 (H-2^d) spleen cell alloantigen IP; they were transferred to new cages and kept in the same room for 3 h to remove the odor of camphor from their coats and then returned to their home room. The NC and US groups were injected at this time with the alloantigen only. For the recall of the response, on day 8 the CND and NC groups were exposed to C for 1 h. The CNDo and US groups were not exposed to camphor odor.

Preparation of Spleen Cells

Animals of each group were killed simultaneously in a box by CO_2 asphyxiation between 0700 and 0730 h. This procedure took only 5–10 min to sacrifice all four groups (CND, CNDo, NC, US). Spleens were removed immediately and placed into individual petri plates containing sterile 0.9% sodium chloride solution (saline) on ice. The spleen cells were expelled from the sac with the help of a forceps and a syringe with 23-ga needle. The single cell suspension was collected with a 23-ga needle and a 3-ml syringe into a sterile 15-ml tube. The tubes were filled with saline and centrifuged at 2000 rpm for 5 min at 5°C in a Beckman centrifuge. The supernatant was discarded, and the washing was repeated once more. The pellet was suspended with 1 ml of sterile saline with a sterile Pasteur pipette to remove the debris. Spleen cell counts were made in a Coulter counter following lysis of red blood cells with saponin. Whole spleen cells (with red blood cells) were used in the CTL and NK cell assay.

Assay for CTL or NK Cell Activity

YC8 and YAC-1 cells served as targets for CTL and NK cells, respectively. These cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 μg streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The YC8 and YAC-1 cells were cultured into fresh tissue culture flasks with fresh medium 24 h before harvesting for the assay. With this procedure, the viability is >95%, and the spontaneous release in the chromium release assay is <5–15%. YC8 and YAC-1 cells were labeled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 μCi/1×10^6 cells in a very small volume (total volume is about 0.2 ml) at 37°C in a CO_2 incubator for 30 min. The cells were washed with a large excess of medium two times and suspended at a final density of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. For the NK cell activity, 0.1 ml of spleen effector cells at ratios of 200 : 1, 100 : 1, 50 : 1, and 25 : 1 (E : T ratio) were mixed in triplicate wells with 0.1 ml of 1×10^4 ^{51}Cr-labeled YAC-1 target cells in 96-well, flat-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). Plates were incubated for 4 h in a humidified, 37°C, CO_2 incubator. For the CTL assay, spleen effector and target cells (YC8) were cocultured at 200 : 1, 100 : 1, 50 : 1, and 25 : 1 for 16 h at 37°C in a humidified CO_2 incubator. Supernatant (0.1 ml) from each well was collected after centrifugation of plates. The radioactivity of the samples was counted in a Beckman gamma counter. Maximum ^{51}Cr released from the target cells (MR) was measured after incubation in the presence of 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) and spontaneous release (SR) in the presence of medium. Percent specific ^{51}Cr release was calculated as 100 × [(test release − SR)/MR − SR].

RESULTS

To develop a conditioning paradigm to demonstrate resistance to YC8 tumor, it was first necessary to examine the kinetics of the primary CTL response to the DBA/2 spleen cell alloantigen injection. Although CTL can be demonstrated in the peritoneal cavity after a single injection of 1.5×10^7 DBA/2 spleen cell alloantigen IP, we were unable to detect significant levels of CTL (Table 1) or NK cell activity (Table 2) in the spleens of animals given primary immunizations. After a primary injection, the CTL activity peaked in the peritoneal cavity on day 4 (data not shown), but the CTL activity in the spleen that peaked between 5 and 8 days was too low to be useful for conditioning studies. The kinetics of NK cell activity was equally unremarkable. Although we expected the NK cell activity to peak early as a result of induction of IL-1 and IL-2 by the alloantigen, this was not observed. Because the number of peritoneal exudate cells that can be recovered would be too low to assess responses made by individual animals, we investigated whether greater activity of CTL could be elicited in mice undergoing a secondary response. The low primary response in BALB/c mice to immunization with DBA/2 spleen cell alloantigens may be due to the fact that both strains possess the same major histocompatibility (MHC) H-2^d class I antigens on their cells.

The kinetics of the secondary response was measured by first injecting a group of mice with 1.5×10^7 cells SC to prime the animals. Priming the animal with DBA/2 spleen cells sensitizes the animal to the minor MHC alloantigens. Thirty days after sensitization, the animals were injected with 1.5×10^7 DBA/2 spleen cells IP (day 0). The immunizations were staggered so that all animals were sacrificed and assayed for splenic CTL activity on the same day with the same target cell preparation. The secondary CTL response peaked between 4 and 6 days and was on its downward slope by day 8 (Table 3).

Based on the kinetics of the secondary response, we devised the conditioning protocol shown in Table 4. In this paradigm, the conditioned mice (CND) received only one CS/US association on day 0, and reexposure to the CS was made on day 8. The results in Table 5 show that only the CND group showed a significant elevation of CTL activity over all of the control groups (CNDo, NC, and US). There were no significant differences among the control groups. Although the effect of the conditioning was small, it was statistically significant and reproducible. Consistent with these observations, we have also shown that the primary CTL response to EL-4 (H-2^b) target cells can be conditioned using C57BL/6 (H-2^b) spleen cell alloantigen as the unconditioned stimulus (11).

DISCUSSION

It has long been known that once a tumor foci develops, the capacity to resist tumor growth dissipates. Besedovsky et al. (3) showed that recognition of tumor antigen by mature T

TABLE 1

KINETICS OF PRIMARY CTL RESPONSE TO DBA/2 SPLEEN CELL ALLOANTIGEN

Days After Immunization	^{51}Cr Released E : T Ratio (%)			
	200 : 1	100 : 1	50 : 1	25 : 1
Not Immunized*	−0.8 ± 1.5‡	−0.7 ± 3.0	−2.4 ± 1.2	−5.5 ± 2.6
1	1.4 ± 1.2	1.2 ± 0.5	1.1 ± 1.9	−0.6 ± 1.1
2	−0.8 ± 2.6	−0.6 ± 0.8	0.6 ± 1.4	−1.1 ± 0.1
4	2.1 ± 1.7	3.0 ± 0.6	0.1 ± 0.7	−1.9 ± 0.4
6	5.8 ± 2.3	6.8 ± 2.2	5.2 ± 2.4	2.1 ± 1.1
8	5.6 ± 3.8	4.1 ± 3.6	1.8 ± 1.0	−1.0 ± 0.7
10	3.7 ± 0.2	3.6 ± 2.6	2.6 ± 0.5	−1.7 ± 1.0

Mice were injected on day 0 with 1.5×10^7 DBA/2 spleen cells IP. There were three mice/group. Target cells were YC8 tumor cells. Values are mean ± SE for the group.

*Injected with saline on day 0.

cells occurs within hours of tumor implantation. A glucocorticoid-increasing factor produced by T-cells stimulated the hypophyseal release of ACTH and corticosterone that suppressed immunity. Gorczynski et al. (8) conditioned immunosuppression and demonstrated its effect on the growth of cancer. They showed that the conditioned suppression was mediated by histamine type 2 receptor bearing suppressor T cells (Ts). It is known that in animals with preexisting tumor, active immunization prolongs survival but rarely produces cures (1,4,5,15,16,22). Attempts to reduce the Ts by injection of cyclophosphamide prior to vaccination to enhance host immunity to cancer have been marginally successful (2,13).

We have used conditioning to increase the effectiveness of vaccine therapy. Our studies suggest that tumor-specific immunotherapy can be combined with conditioning in tumor-bearing animals. BALB/c mice with YC8 lymphoma were conditioned by exposure to the CS plus immunization with DBA/2 spleen cell alloantigen. DBA/2 spleen cells share a common alloantigen(s) with the YC8 tumor. Tumor-bearing animals that received multiple conditioning trials and continued to receive exposure to camphor odor alone at weekly intervals resisted tumor growth. Mice conditioned three or four times showed greater resistance than mice that were similarly conditioned but not exposed to the CS (CNDo). Control mice exposed to the CS only did not resist tumor growth. These studies suggest that a specific immune response can be focused through the CNS to bring resistance to, and in some cases, regression of, a growing neoplasm in vivo (6). Furthermore, when conditioning is done in conjunction with adoptive chemoimmunotherapy, a greater incidence of regressions and cures was observed (7).

The YC8 model is advantageous in that tumor-specific resistance can be established against a syngeneic tumor by injection of a naturally available DBA/2 spleen cell alloantigen. Protective resistance against SC tumor challenge of 5×10^4 YC8 is observed within 10 days after a single IP injection of DBA/2 spleen alloantigen. Although CTL was not generated in the spleen cell compartment within this time frame, noncytolytic spleen cells are able to confer resistance by adoptive transfer. Direct assessment of this primary resistance could not be made by measurement of CTL or NK cell activity because neither of these "read out" systems produced sufficient activity to be accurately measurable in individual animals that were injected once only with the alloantigen. Therefore, an alternate approach was taken to test the conditionability of BALB/c mice to resist the syngeneic YC8 tumor growth in

TABLE 2

KINETICS OF NK CELL RESPONSE TO DBA/2 SPLEEN CELL ALLOANTIGEN

Days After Immunization	^{51}Cr Released E : T Ratio (%)			
	200 : 1	100 : 1	50 : 1	25 : 1
Not Immunized*	1.2 ± 0.6	0.2 ± 0.6	−0.03 ± 0.7	−0.7 ± 0.3
1	1.3 ± 0.1	0.3 ± 1.9	0.7 ± 0.6	−0.3 ± 0.4
2	2.5 ± 1.4	2.3 ± 0.9	1.9 ± 0.3	−0.4 ± 0.4
4	2.2 ± 1.0	1.4 ± 0.4	0.7 ± 0.7	−1.8 ± 0.8
6	5.5 ± 1.5	4.2 ± 1.5	2.8 ± 0.5	1.8 ± 1.4
8	2.2 ± 0.7	2.1 ± 0.6	1.5 ± 0.9	−0.1 ± 0.2
10	3.0 ± 0.2	2.3 ± 0.6	2.7 ± 1.4	0.7 ± 0.2

Mice were injected on day 0 with 1.5×10^7 DBA/2 spleen cells IP. There were three mice/group. Target cells were YAC-1 tumor cells. Values are mean ± SE for the group.

*Injected with saline on day 0.

TABLE 3

KINETICS OF SECONDARY CTL RESPONSE TO DBA/2 SPLEEN CELL ALLOANTIGEN

Days After Immunization	^{51}Cr Released E : T Ratio (%)			
	200 : 1	100 : 1	50 : 1	25 : 1
Not Immunized*	0.4 ± 0.1	1.7 ± 1.5	−1.2 ± 1.4	−3.1 ± 0.4
4	46.0 ± 0.8	47.0 ± 3.6	30.4 ± 0.2	18.3 ± 0.9
6	40.6 ± 0.6	44.6 ± 3.4	31.5 ± 2.8	15.0 ± 0.5
8	24.0 ± 9.0	22.6 ± 4.7	17.5 ± 4.7	8.2 ± 2.2
10	13.0 ± 9.1	13.8 ± 9.1	11.7 ± 5.8	4.1 ± 1.0

Mice were injected on day 0 with 1.5×10^7 DBA/2 spleen cells IP. There were three mice/group. Target cells were YC8 tumor cells. Values were mean ± SE for the group.
*Injected with saline on day 0.

vivo. We developed a single trial paradigm to condition the secondary response in primed animals. The conditioned elevation of the secondary CTL response was low but measurable at different effector to target cell (E : T) ratios. Greater statistical significance was observed at the lower E : T ratios by Student's *t*-test, indicating that the conditioned group (differential response between CND vs. controls) had higher activity at lower E : T ratios. In support of these observations, we have similarly conditioned the primary CTL response using C57BL/6 (H-2^b) spleen cell alloantigens as unconditioned stimulus (11).

We have noted that different cohorts of animals received from the vendor at different times could show variations in their activities. Therefore, sometimes it is difficult to make direct comparisons of CTL activities measured in different cohorts of animals, particularly if the experiments are carried out at different times, for example, the CTL actitvity of spleen cells of the 8- and 10-day groups of Table 3 had larger ranges than the values of Table 5. At least two factors might have contributed to the wider range of activities: 1) a fewer number of animals were used in Table 3, and 2) different batches of animals were used for these two experiments.

The functional interconnection between the CNS and immune system (IS) has long been known but the specific mechanisms through which the immune system and CNS communicate has not been clarified. It is essential that these pathways be delineated if we are to understand the role of the CNS in health and disease. Conditioning of NK cell activity was used to probe for pathways of communication between the CNS and IS. Our studies suggest that during the CS/US association the NK cell system communicates with the CNS through interferon-β (IFN-β). A series of evidence supports this contention. 1) IFN-β can replace poly I : C as the US (18). 2) The conditioned response can be blocked if, prior to the CS/US association, rabbit anti-IFN antiserum (100 neutralizing units) is injected into the cisterna magna (CM). This implies that IFN-β, which reaches the CNS by way of the circulation, is neutralized centrally, thus preventing the CS/US learning from taking place. 3) Injection of 100 IU of IFN-β SC or IV, an amount not sufficient to raise the NK cells in the periphery, when injected directly into the CM following exposure to camphor is able to condition the NK cells response (20). These results, taken together, suggest that during the CS/US pairing the CNS "reads" the IFN-β message and stores the association in memory. On subsequent exposure, the CS recalls from memory the stored information and triggers the NK cell response. During the recall from memory of the conditioned response, an opioid-mediated pathway is utilized centrally (21), and that such an effector pathway exists centrally was directly tested by injection of Met-enkephalin (opioid) directly into the CM of mice for which NK cells were preactivated with

TABLE 4

PROTOCOL FOR CONDITIONING THE SECONDARY CTL RESPONSE

Group	*n*	Days		
		0	8	9
CND	11	C+D	C	CTL
CNDo	11	C+D		CTL
NC	11	D	C	CTL
US	11	D		CTL

Mice were primed with DBA/2 spleen cell alloantigen SC 30 days before being used for the conditioning trial. On day 0, mice in the conditioned (CND) and CNDo groups were exposed to the odor of camphor (C) for 1 h after which they were immediately given an injection of 1.5×10^7 DBA/2 spleen cell alloantigen IP (D). Mice in the nonconditioned (NC) and unconditioned stimulus (US) groups were injected with D only on this day. On day 8, at a time when the CTL response was on a downward slope, mice in the CND and NC groups were exposed to C. CNDo and US groups were not exposed to C. Spleen cells were assayed for CTL activity on day 9.

TABLE 5

CONDITIONED SECONDARY SYNGENEIC CTL RESPONSE AGAINST YC8 TUMOR CELLS

Group	*n*	^{51}Cr Released E : T Ratio (%)			
		200 : 1	100 : 1	50 : 1	25 : 1
CND	11	14.1 ± 1.7	11.3 ± 1.4	14.2 ± 2.2	7.1 ± 1.8
CNDo	11	11.1 ± 1.5	9.5 ± 1.5	9.8 ± 2.0	2.2 ± 0.8
NC	11	8.7 ± 1.2	6.5 ± 1.2	5.1 ± 1.0	−0.5 ± 0.8
US	11	12.3 ± 1.5	8.0 ± 1.3	6.6 ± 1.0	2.6 ± 0.6

Values are mean ± SE for the group. Overall comparison of groups using repeated-measures ANOVA with Duncan's Multiple Range test, $F(3, 40) = 5.37$, $p = 0.0033$, pairwise comparison of CND vs. CNDo, NC, or US is significant, $p < 0.05$. CNDo, NC, and US were not significantly different.

suboptimal dose of 1 μg poly I : C (14). These studies with the NK cell model (12,14,18,21) have provided insight into the pathway of communication between the immune system and CNS and it has been reviewed (9).

The CTL response is a MHC-restricted specific response that does not involve production of IFN-α, β. Consequently, it is unlikely that IFN-α, β would be the mediators required for learning this response. However, IFN-γ or other cytokines (IL-1, IL-2, TNF-α) that are produced in response to immunization with the alloantigen might be likely candidates. Our studies (19,20) suggest that it may be possible to identify the mediators induced by the US that are involved in the conditioned response. Therefore, conditioning might provide a way to investigate other pathways of CNS–organ system communications, with the caveat that the approach may not be applicable to all forms of conditioning.

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