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Conditioning of the Allogeneic Cytotoxic Lymphocyte Response

RAYMOND N. HIRAMOTO,[1] CHI-MEI HSUEH, CAROLYN F. ROGERS, SOSSIENA DEMISSIE, NANCY S. HIRAMOTO, SENG-JAW SOONG AND VITHAL K. GHANTA

Departments of Microbiology, Biology, and Biostatistics, University of Alabama at Birmingham, University Station, Birmingham, AL 35294

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HIRAMOTO, R. N., C.-M. HSUEH, C. F. ROGERS, S. DEMISSIE, N. S. HIRAMOTO, S.-J. SOONG AND V. K. GHANTA. *Conditioning of the allogeneic cytotoxic lymphocyte response.* PHARMACOL BIOCHEM BEHAV 44(2) 275-280, 1993.—Allogeneic cytotoxic T-lymphocyte (CTL) response can be obtained following immunization of BALB/c mice with C57BL/6 spleen cells. We investigated the possibility of behaviorally conditioning this response by associating the C57BL/6 spleen cell immunization [unconditioned stimulus (US)] with camphor odor [conditioned stimulus (CS)]. We reported the possible mechanisms involved in the conditioning of natural killer cell activity. Similar approaches were used to investigate the mechanisms that participate in the conditioned CTL activity. The first mechanism of investigation utilized opioid receptor blockers naltrexone and quaternary naltrexone. Naltrexone, which blocks both the central and peripheral opioid receptors, blocked the recall of the conditioned response, whereas quaternary naltrexone, which does not penetrate the blood-brain barrier, was unable to block the conditioned response, demonstrating that centrally located opioid receptors play a role in the recall of the conditioned response. The studies are of interest because they indicate that resistance or susceptibility to various diseases such as cancer, autoimmunity, and infectious diseases might be influenced by the regulatory network of the CNS.

CTL response   Conditioned response   Opioid receptor blockers   Naltrexone and quaternary naltrexone

A large variety of organs and tissues are grafted both clinically and experimentally, but because in most cases the grafted tissues are not autologous potential allograft rejection responses are controlled by the use of immunosuppressive drugs. Potential effector mechanisms for allograft rejections are by cytotoxic T-lymphocytes (CTLs) and/or delayed type hypersensitivity (DTH) reactivity response mediated by $CD4^+$ T-cells (15,17,20). The CTLs are thought to be specific for their capacity to bind to the relevant target cells (4,5). The activities of CTLs are major histocompatibility (MHC) restricted in that they are directed against cells bearing the same class I MHC antigens as the antigen presenting cells (APCs) with the original immunogen (1,6,19). The CTL response is a specific response and it is probable that alloreactive T-cells belong to the same lineage as antigen-specific cells (14,18).

Our studies focused on conditioning the natural killer (NK) cell activity (22) and fever (11). Both these responses, although nonspecific, are important as part of the host defense against foreign agents (viruses, bacteria, fungi, etc.). We conditioned a specific CTL activity against an allogeneic target. Because fever, NK cell activity, and specific CTL responses can be activated by the CNS, it indicates that the CNS, under proper stimulus, might be able to augment host defense and immunity to foreign antigens and autologous tumor-specific antigens (16). On the other hand, a potential negative downside is that signals to the CNS might play a role in rejecting a heart, kidney, or marrow allograft critical for host survival.

Our studies show that CTL response can be conditioned by pairing camphor odor [conditioned stimulus (CS)] with injection of C57BL/6 spleen cells [unconditioned stimulus (US)]. CS/US association was necessary for the CS to evoke the conditioned response, that is, the enhancement of CTL activity in the spleen of allogeneic BALB/c mice.

METHOD

Mice

Six-week-old female BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, DE) and maintained on standard rodent chow and water ad lib with a 12 L : 12 D cycle. Lights were on at 7:00 a.m. and off at 7:00 p.m. Animals were allowed to adapt to our vivarium surroundings for at least 1 week before use in the experiments. Drugs used for this study were naltrexone (Ntx) (Sigma Chemical Co., St. Louis, MO) and quaternary naltrexone, a gift of Dr. Merz, Boehringer-Ingelheim, West Germany (25).

[1] To whom requests for reprints should be addressed.

Conditioning the CTL Response

All conditioning procedures were started between 7:00 and 7:30 a.m. Exposure to the odor stimulus and treatment with US were completed by 8:30 a.m. Procedures were performed as soon as lights were on to accommodate the diurnal cycle and low corticosteroid levels. A 1-oz. block of camphor (C) was partially dissolved in mineral oil (1 block to about 150 ml mineral oil) while stirring on low heat. C exposure was carried out inside a cabinet in a different laboratory. Thirty milliliters of the camphor/mineral oil mixture 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 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 control animals.

Mice of each group were housed separately in individual cages of 10/cage for 1 week prior to the performance of the experiment. On day 0, the conditioned (CND) and CNDo groups were moved to the room with the cabinet and exposed to the odor of camphor for 1 h without food and water. After camphor exposure, animals were injected with 1.5×10^7 C57BL/6 spleen cell alloantigen IP, transferred to new cages, and kept in the room for 3 h to remove camphor odor from their coats before returning to their homeroom. The nonconditioned (NC) and US groups were injected at this time with the alloantigen only. For recall of the response, on day 6 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 with CO_2 asphyxiation. Animals were sacrificed before 8:00 a.m. This procedure took only 5–10 min to sacrifice all four groups (CND, CNDo, NC, and 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 spleen sac with the help of a forceps and needle. The single-cell suspension was collected with a 23-ga needle and 3-ml syringe into a sterile 15-ml tube. Tubes were filled with saline and centrifuged at 2,000 rpm for 5 min at 5°C in a Beckman centrifuge (Beckman Instruments, Fullerton, CA). The supernatant was discarded and the washing was repeated once more. The pellet was suspended with 1 ml sterile saline using 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 assays.

Assays for CTL and NK Cell Activity

EL-4 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. EL-4 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 <10%. EL-4 and YAC-1 cells were labeled with sodium chromate (Amersham Corp., Arlington Heights, IL) at a ratio of 100 μCi/1×10^6 cells in a total volume of 0.2 ml at 37°C in a CO_2 incubator for 30 min. The cells were washed with a large excess of medium 2× and suspended at a final density of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. One tenth milliliter 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 1×10^4 ^{51}Cr-labeled EL-4 or YAC-1 target cells in 96-well, flat-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). Plates were incubated for 4 h for NK cell activity and 20 h for CTL assay in a humidified, 37°C, CO_2 incubator. One-tenth milliliter of supernatant 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) and spontaneous release (SR) in the presence of medium. Percent specific ^{51}Cr release was calculated as 100× [(test release-SR)/MR-SR].

RESULTS

Kinetics of the CTL and NK Cell Response

Initial studies were done to establish the time when CTL response was maximum in the spleen. Groups of BALB/c mice (n = 3) were immunized with 1.5×10^7 C57BL/6 normal spleen cells IP. The immunization was staggered so that all animals were sacrificed on the same day and spleen cells from each animal were tested for CTL and NK cell activities individually. The results show that no CTL activity was discernable in animals sacrificed at 1 or 2 days after immunization when compared with the unimmunized control mice (Table 1). A peak CTL response was recorded on day 4 that declined by day 6 and still remained detectable at day 10. The

TABLE 1

KINETICS OF ALLOGENEIC CYTOTOXIC T-LYMPHOCYTE RESPONSE

	% ^{51}Cr Released E : T Ratio			
Days Postimmunization	200 : 1	100 : 1	50 : 1	25 : 1
1	−4.8 ± 0.5*	−3.0 ± 0.4	−2.4 ± 0.4	−3.7 ± 0.8
2	−6.3 ± 0.7	−3.8 ± 1.2	−3.4 ± 0.3	−4.5 ± 0.6
4	44.5 ± 3.8	34.6 ± 4.6	22.4 ± 3.1	11.8 ± 1.8
6	24.5 ± 2.3	16.7 ± 1.3	8.6 ± 1.4	2.1 ± 1.7
10	20.6 ± 5.4	15.0 ± 3.5	8.0 ± 2.2	2.6 ± 0.3
Not immunized	−5.3 ± 0.5	−2.8 ± 0.2	−2.9 ± 0.4	−3.5 ± 0.8

*Values were mean for the group ± SE.

TABLE 2

KINETICS OF NK RESPONSE

Days Postimmunization	% ^{51}Cr Released E : T Ratio 200 : 1	100 : 1	50 : 1	25 : 1
1	0.8 ± 0.2*	0.8 ± 0.3	0.7 ± 0.3	0.2 ± 0.2
2	1.4 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	0.3 ± 0.0
4	3.2 ± 0.5	3.1 ± 0.2	1.8 ± 0.2	0.4 ± 0.2
6	1.1 ± 0.2	0.8 ± 0.3	0.7 ± 0.1	−0.2 ± 0.3
10	1.4 ± 0.6	1.1 ± 0.2	0.2 ± 0.1	−0.7 ± 0.2
Not immunized	1.1 ± 0.1	1.2 ± 0.2	0.5 ± 0.1	0.1 ± 0.2

*Values were mean for the group ± SE.

spleen cells of each animal were also tested for NK cell activity. It was of interest to determine the effect of alloimmunization on activation of NK cell activity. NK cell activity remained low throughout the entire course during which CTL activity was being activated (Table 2). NK cell activity was tested using YAC-1 target cells. The fact that no NK cell activity could be demonstrated indicates that even during the peak generation of CTL sufficient interferon (IFN)-α or -β was not produced to elevate NK cell activity in the spleen.

Conditioning of the CTL Response

A short paradigm was devised to see if CTL response can be conditioned. The paradigm was based upon the observation that the alloimmune CTL response peaks with day 4 and begins to decline by day 6; therefore, testing for the conditioned response after day 6 will allow us to see if conditioned elevation of the CTL can be evoked by signals transmitted to the CNS. The conditioning protocol is shown in Table 3. The US group served as a control for the response to the immunization. Mice in all groups were assayed for CTL activity on day 7 (Table 4).

The conditioned group showed an elevation of CTL activity. The average CTL activity at 200 : 1 was 12.4%. The range of activity was 35.2–1.3%, indicating that all animals were not conditioned. The control groups CNDo, NC, and US showed lower mean CTL activity than the CND group at all E : T ratios. The CND group showed statistically higher CTL activity over the CNDo and US groups (p = 0.0106 and 0.0024, respectively) but was not significant over NC (p = 0.0638). In light of these results, the studies were repeated using the same protocol in which a CND group was compared against an NC group (Table 5). Once again, the mean CTL activity in the CND group was elevated over the NC group. The activity in the CND and NC groups were proportionately higher than the cohorts tested in Table 3. The reason for this difference, frequently observed in different cohorts of animals tested at different times, is not clear, but despite these differences in the control CTL activity the elevation of CTL activity in the conditioned group could be readily demonstrated (CND vs. NC, Experiment 1, p = 0.0168; Experiment 2, $p < 0.05$).

Effect of Ntx on the Conditioned Response

Previous studies in our laboratory have shown that the CS/US association is not interrupted by prior treatment of the animals by Ntx, but the expression of the conditioned response could be blocked by Ntx (25). To test whether the pathways of the conditioned CTL response mimic that of the NK cell response, a Ntx blocking experiment was done as follows. Four groups of mice were compared. The CND and CNDntx mice were conditioned on day 0 by exposure to camphor followed by injection of C57BL/6 spleen cells. Mice in the NC and NCntx groups were injected with the C57BL/6 spleen cells only. On day 6, mice in the CND and NC groups were injected IP with 0.1 ml saline and mice in the CNDntx and NCntx with 10 mg/kg naltrexone IP. Animals were rested for 10 min, followed by exposure to camphor for 1 h. The results are shown in Table 6. Only mice in the CND group showed a conditioned elevation of the CTL activity. The response in the CND group was statistically significant over the NC group. The CND and NC groups served as positive controls, showing that the conditions for conditioning were reliably reproduced. Comparison of the CNDntx vs. NCntx

TABLE 3

PROTOCOL FOR CONDITIONING THE CYTOTOXIC T-LYMPHOCYTE RESPONSE

Groups	Days 0	1	2	3	4	5	6	7
CND	C* + C57†	–	–	–	–	–	C	CTL
CNDo	C + C57	–	–	–	–	–	–	CTL
NC	C57	–	–	–	–	–	C	CTL
US	C57	–	–	–	–	–	–	CTL

*C, exposure to camphor odor in an enclosed cabinet for 1 h.

†C57, injection with 1.5 × 10^7 cells/0.1cc IP allogeneic C57BL/6 spleen cells immediately after removal from camphor.

TABLE 4

CONDITIONING THE CYTOTOXIC T-LYMPHOCYTE RESPONSE

Group	n	% ^{51}Cr Released E : T Ratio 200 : 1	100 : 1	50 : 1	25 : 1
CND	10	12.4 ± 3.1*	6.4 ± 2.5	3.6 ± 1.9	2.5 ± 1.4
CNDo	10	6.3 ± 1.7	0.04 ± 1.0	−3.0 ± 0.9	5.9 ± 0.5
NC	10	5.4 ± 1.5	3.9 ± 1.0	0.6 ± 0.6	−3.5 ± 0.7
US	10	3.0 ± 1.8	−1.2 ± 1.2	−4.2 ± 1.2	−7.5 ± 0.8

*Values were mean for the group ± SE. Values of the CND group were compared with CNDo, NC, and US groups for similarities and differences by repeated-measures ANOVA with an α value of 0.05. The p values of comparisons were CND vs. CNDo, $F(1, 18) = 8.13$, $p = 0.0106$; CND vs. NC, $F(1, 18) = 3.9$, $p = 0.0638$; CND vs. US, $F(1, 18) = 12.51$, $p = 0.0024$.

showed that treatment with naltrexone blocks the learned response, just as, for the expression of the NK cell response (25), opioid receptors are needed for the recall phase of the CTL response.

Effect of Quaternary Ntx on the CTL Response

Naltrexone blocks central and peripheral opioid receptors. On the other hand, quaternary Ntx (Qntx) cannot penetrate the blood–brain barrier and only opioid receptors located in the periphery are effectively blocked. To assess the effect of Qntx, the same experimental design was used as for the naltrexone experiment except Qntx (10 mg/kg, IP) replaced Ntx. We pretested Qntx at this dose and have shown that it specifically blocks in vivo the NK cell response to met-enkephalin (unpublished observation). The results of this study are shown in Table 7. The results show treatment of conditioned animals with Qntx did not prevent recall of the conditioned response. The CNDqntx group demonstrated that it was conditioned over the NCqntx group. These results indicate that the inhibition of the conditioned response observed with Ntx was due to blocking of opioid receptors located within the CNS. Therefore, just as with the NK cell system, a central opioid receptor-mediated pathway is involved in activating the sequence that permits conditioned response from memory.

DISCUSSION

In previous studies we have shown that elevation of NK cell activity (11,12,22,25), elevation of core body temperature (11), and resistance to growth of cancer cells in vivo (8) can be conditioned. These results imply that a CNS-directed immune activity can become established with proper training of animals under specifically controlled experimental conditions. Moreover, the afferent signal from the immune system that allows CS/US associations to occur within the CNS has been recently identified as IFN-β for the NK cell response (23). Other studies in rats and mice have shown modification of T-cell subset ratios (13) or activities (9) can be conditioned, although the specific signals to the CNS have not been identified.

The mechanisms by which resistance is brought about through the CNS against allografts still remain to be identified. In our studies, we attempted to combine camphor odor with allogeneic immunization to demonstrate that specific MHC-restricted cytotoxic lymphocyte activity can also become conditioned. Previously, we used an allogeneic vaccine to immunize BALB/c mice against the syngeneic YC8 lymphoma and have shown that conditioned resistance to the tumor can be established in vivo. The current results support the view that conditioned immune (specific) resistance to cancer might have a cellular (CTL) basis for its mechanism. In the present study, we have shown that when BALB/c mice were injected with allogeneic spleen cells the CTL activity peaked at about 4 days but NK cell activity remained low during the course of the CTL response. This was surprising in light of the fact that allogeneic antigen should have activated macrophages and T-helper cells, which in turn should have produced physiological levels of interleukin (IL)-1 and IL-2,

TABLE 5

CONDITIONING THE CYTOTOXIC LYMPHOCYTE RESPONSE

Group	n	% ^{51}Cr Released E : T Ratio 200 : 1	100 : 1	50 : 1	25 : 1
Experiment 1					
CND	10	28.7 ± 2.7*	27.7 ± 3.1	18.3 ± 2.1	11.0 ± 2.0
NC	10	22.6 ± 1.6	20.4 ± 2.1	11.1 ± 1.1	3.7 ± 1.3
Experiment 2					
CND	10	25.8 ± 3.9	20.6 ± 3.0	14.1 ± 2.6	9.7 ± 1.7
NC	10	16.3 ± 3.3	11.9 ± 2.9	6.9 ± 1.9	4.1 ± 1.5

*Values were mean for the group ± SE. The two groups (CND and NC) were compared by repeated-measures ANOVA with an α value of 0.05 for the groups similarities and differences in activities. Experiment 1, CND vs. NC, $F(1, 18) = 6.94$, $p = 0.0168$; Experiment 2, CND vs. NC, $F(1, 18) = 4.94$, $p = 0.0393$.

TABLE 6

NALTREXONE BLOCK OF THE CONDITIONED ALLOGENEIC CTL RESPONSE

Group	n	% ^{51}Cr Released E : T Ratio 200 : 1	100 : 1	50 : 1	25 : 1
CND	10	8.04 ± 1.06*	6.70 ± 1.04	1.54 ± 0.81	−1.42 ± 0.82
NC	10	1.79 ± 1.44	0.50 ± 1.09	−1.88 ± 1.02	−6.40 ± 0.95
CNDntx	10	4.17 ± 0.96	0.53 ± 0.64	−1.01 ± 0.60	−3.43 ± 0.84
NCntx	10	1.78 ± 1.08	−0.4 ± 1.0	−0.11 ± 0.80	−3.64 ± 0.47

*Values were mean for the group ± SE. Statistical comparisons were made between groups by repeated-measures ANOVA with an α value of 0.05. CND differed significantly from NC, CNDntx, and NCntx [CND vs. NC, $F(1, 18) = 18.93$, $p = 0.0004$; CND vs. CNDntx, $F(1, 18) = 13.04$, $p = 0.002$; CND vs. NCntx, $F(1, 18) = 16.12$, $p = 0.0008$]. Conditioned group treated with naltrexone did not differ from NCntx and NC [CNDntx vs. NCntx, $F(1, 18) = 0.34$, $p = 0.565$; NC vs. CNDntx, $F(1, 18) = 2.12$, $p = 0.1628$]. The NC and NCntx groups did not differ from each other [NC vs. NCntx, $F(1, 18) = 0.88$, $p = 0.3612$].

both of which are necessary for the induction of the CTL response. The induction of these cytokines should have enhanced NK cell activation in the spleen (2,7). The fact that NK cell activity remained at a nearly background level indicates that perhaps these mediators are not the primary stimulants for NK cell activation in vivo.

Based upon the kinetics of the CTL response, we devised a short paradigm to test if CTL activity can be conditioned. The paradigm is simple in that only one CS/US association is made on day 0 and only one exposure to CS is given on day 6 to recall the conditioned response. The studies show that in the CND group the CS/US linkage had been established because only the conditioned group demonstrated an elevation of CTL activity upon reexposure to the CS alone. In the control groups (CNDo, NC, and US), no conditioned response was observed. We have also shown for the NK cell model that if the US is given before the CS (backward conditioning) the conditioned NK cell response is not learned (10). While conditioning of the CTL response could be readily and repeatedly demonstrated from experiment to experiment, a point for concern was that the strength of the CTL response on day 6 was not consistently reproduced from experiment to experiment. The reason for this is not clear. For example, in Table 4 the US group was given alloantigen only and by day 7 its CTL response was only 3% at E : T ratio 200 : 1, comparable to that seen for CNDo and NC but not for day 6 or day 10 animals in Table 1. Whether the CTL response in normal mice in some cohorts reaches low values due to a rapid decline by day 7 of the CTL response or if the peak response on day 4 in these cohorts was low to begin with is not known.

The studies with naltrexone and quaternary naltrexone point to a recall pathway in the CNS for the conditioned response that depends upon the presence of opioid receptors. In this regard, the observation was similar to that seen for the conditioned response reported for the NK cell system (25). Moreover, the fact that the peripheral block of opioid receptors did not inhibit the CTL response indicates that nonopioid neuroendocrine mediators might also be involved in signaling the activation of CTL in the spleen.

The olfactory and immune systems both have similarities in having the ability of "self-marking," that is, recognition of self from nonself. Moreover, both systems are critical for host survival. It has been speculated by Thomas (27) that both systems might have evolved from a single ancestral system employed early on in evolution and that H-2, which dictates immune responses, also dictates pheromones, which a female uses to distinguish males of different strains (21,26,28,29). Tracking of male pheromones from stud mice is described as the Bruce effect and is important in sustaining pregnancy (3). We speculate that throughout the evolutionary period both the immune and olfactory systems might have interacted intimately through the CNS. This view is supported by evidence that multiple conditioning trials were not required to condition the NK, fever, and CTL responses. Conditioning can be accomplished with only one CS/US association (11,24). The need for multiple trainings as is required for certain conditioning paradigms implies that the pathway for associating the CS with the US had not been preestablished and the need for learning of the response may not be as critical for survival.

In the NK model, the interstimulus interval can be sepa-

TABLE 7

EFFECT OF QUATERNARY NALTREXONE ON THE CONDITIONED ALLOGENEIC CTL RESPONSE

Group	n	% ^{51}Cr Released E : T Ratio 200 : 1	100 : 1	50 : 1	25 : 1
CNDqntx	9	25.5 ± 3.6*	22.3 ± 3.2	11.1 ± 1.6	0.6 ± 1.3
NCqntx	9	14.1 ± 2.3	13.3 ± 2.1	5.9 ± 2.1	−1.1 ± 1.6

*Values were mean for each group ± SE. Statistical comparison was made with repeated-measures ANOVA with an α value of 0.05, $F(1, 16) = 5.58$, $p = 0.0311$.

rated by as long as 2 days and conditioning can still be accomplished (10). The fact that learning still takes place even when the CS is separated from the US by 2 days indicates the CNS must be keenly aware of incoming olfactory signals and is able to link these events even when the signals from the immune system are delayed by 2 days. This implies that processing is taking place unconsciously, that is, without the host perceiving the events. If this is true, then the CS/US association is being made involuntarily by the CNS and training under anesthesia should be possible. We successfully conditioned the NK cell response in anesthetized mice (unpublished observation). Together, these observations point to the long-established communication between the olfactory and immune systems and the singular importance of this communication for survival of the species. Thus, the employment of the olfactory cues to condition resistance to cancer and other diseases might have advantages in terms of simplicity over other paradigms that require conscious awareness (imagery) of the CS and US signals being employed.

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