Acquisition and extinction of conditional immunoenhancement following training with cyclophosphamide

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Paired-group mice were injected with pentobarbital (a pharmacological conditioned stimulus, CS) prior to injection of cyclophosphamide (Cy), an immunosuppressive drug. Unpaired-group mice received Cy 24 h after the CS. CS-alone mice received only pentobarbital. When reexposed to the CS in conjunction with an antigenic challenge, unpaired-group mice displayed suppressed antibody titers, while paired-group mice displayed titers similar to those of CS-alone mice. Thus, the residual immunosuppressive effect of Cy was eliminated by the Cy-paired cue. Subsequently half the mice in each group were repeatedly presented with the CS and half were left undisturbed. Mice were again challenged and reexposed to the CS. Unpaired-group mice now had titers equivalent to CS-alone mice, indicating that the immunosuppressive effect of Cy had dissipated. Results from paired-group mice indicated a frank conditional immunoenhancement that was extinguished by repeated CS presentations.

Cyclophosphamide (Cy) is a potent immunosuppressive drug. Ader and Cohen (1975), for example, reported that rats challenged with an antigen (sheep red blood cells, SRBCs) 3 days after a single Cy injection displayed significantly lower hemagglutinating antibody titers than rats not administered Cy. Ader and Cohen (1975) also reported that this immunosuppressive effect was subject to Pavlovian conditioning. After a single, paired presentation of saccharin (SAC) and Cy, reexposure to SAC following the antigenic challenge further compromised immunological functioning. Subsequent research by Ader and Cohen and their colleagues (see Ader & Cohen, 1985), as well as by others (e.g., Rogers, Reich, Strom, & Carpenter, 1976; Wayner, Flannery, & Singer, 1978) has confirmed Ader and Cohen's (1975) results. That is, antibody titers seen in conditioned rats are even lower than those seen in nonconditioned rats (e.g., rats with a preimmunization history of SAC and Cy presented in a noncontingent manner). Similar findings have been reported for mice (e.g., Gorchynski, MacRae, & Kennedy, 1984). The compromised immunological functioning seen in nonconditioned animals results from the residual immunosuppressive effects of Cy administered some days before SRBC challenge and SAC reexposure. The further immunosuppression seen in conditioned animals is assumed to result from the augmentation of the residual unconditional effect of Cy by a SAC-elicited immunosuppressive conditional response (CR).

Bovbjerg, Ader, and Cohen (1984) demonstrated that SAC-elicited conditional immunosuppression can be extinguished by repeated SAC presentations. In this experiment, immunocompetency was assessed with a graft-versus-host response (GvHR) induced by immunizing subjects with splenic leukocytes. The immunosuppressive CR was assessed by the magnitude of the GvHR when animals, following a single SAC-Cy pairing, were reexposed to SAC at the time of immunization. Conditioned rats that had little or no exposure to SAC between the conditioning and immunization phases of the experiment displayed relatively minor GvHRs. That is, they displayed compromised immunological functioning. However, animals with many SAC presentations subsequent to the SAC-Cy pairing displayed no evidence of conditional immunosuppression. Thus, repeated presentations of the flavor conditional stimulus (CS) eliminated the ability of the cue to elicit an immunosuppressive CR.

Some investigators have suggested that conditional impairment of immunological activity is enigmatic, in view of data and theories concerning the adaptive nature of CRs. That is, it has been hypothesized that CRs provide a mechanism with which organisms are better able to deal with environmental demands (see, e.g., Pavlov, 1927, p. 14; Hollis, 1982) or adapt to repeated stimulation (see, e.g., Siegel, Krank, & Hinson, 1987; Wagner, 1976). Some investigators have suggested that it is difficult to suggest an adaptive process conferred by CS-elicited immunosuppression in an organism confronted with an antigenic challenge (e.g., Greenberg, Dyck, & Sandler, 1984; Hinson, 1985; Krank, 1985; Revusky, 1985; Siegel et al., 1987; Weiss, 1985).¹

Reports that Cy-paired CSs elicit Cy-like immunosuppression also appear contrary to influential analyses of

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pharmacological conditioning (Eikelboom & Stewart, 1982; Matysiak & Green, 1984; Obál, 1966; Stewart & Eikelboom, 1987). In these analyses, drug effects mediated by the central nervous system have been distinguished from those involving direct alteration in peripheral homeostasis. In the latter case, the conditioned drug response would be expected to compensate for the drug effect (rather than mimic it). The immunosuppressive effect of Cy is likely due to direct effects on the cells of the immune system, without the participation of the central nervous system (see Klosterhalfen, 1989; Krank & MacQueen, 1988); thus, it would be expected that the Cy-anticipatory CR would compensate for the drug's immunosuppressive effect.

Indeed, in contrast with results indicating that a Cy-paired CS enhances the residual immunosuppressive effects of Cy are results indicating that such a CS elicits a compensatory *reduction* in such immunosuppression. Górczynski et al. (1984), in addition to reporting evidence for conditional immunosuppression, also reported that some conditions favor the expression of *antisuppressive* CRs. That is, conditioned mice display little or no immunosuppression when reexposed to the CS in conjunction with antigenic challenge. This is evidence of an *antisuppressive* CR, since nonconditioned animals (with a prechallenge history of Cy administration the same as that of paired animals) display immunosuppression resulting from the persistent effects of Cy. Similar results have been obtained in more recent experiments with mice (Krank, 1989; Krank & MacQueen, 1988) and with rats (MacQueen & Siegel, 1989).

A complication in most studies of Cy conditioning, especially those demonstrating compensatory conditioning, concerns the suppressed level of immunological activity in control groups as a result of the long-lasting effect of Cy. Thus, results from conditioned animals typically are compared with those obtained from nonconditioned but immunosuppressed animals.² All reports of Cy-compensatory conditioning have inferred such conditioning because conditioned animals failed to display immunosuppression, but there have been no demonstrations of actual immunoenhancement in response to the CS. Many drug-compensatory CRs have been revealed as CS-elicited responses clearly opposite to the drug effect (Siegel, 1989). If some procedures favor the expression of a Cy-compensatory CR, such a CR should be demonstrable by immunomodulation opposite to that induced by Cy. That is, conditioned animals might be expected to display *immunoenhancement* if they are presented with the CS when they are no longer immunologically compromised. The design of the present experiment permitted evaluation of the immunological CR long after the last Cy administration, when no residual immunosuppressive effect of Cy would be expected.

In addition, the design of the present experiment permitted evaluation of extinction of the immunological CR seen after training with Cy. If the CS-elicited alteration

in immune functioning results from Pavlovian conditioning, it should be attenuated by repeated presentation of the CS without Cy (see Bovbjerg et al., 1984).

The factors favoring the expression of either the immunosuppressive or the anti-immunosuppressive CR following training with Cy are unclear. Krank and MacQueen (1988) and Krank (1989) suggested that suppressive CRs are likely to be obtained with gustatory CSs (such as SAC), and that antisuppressive CRs are likely to be obtained with nongustatory CSs (such as environmental cues).³ In accordance with this suggestion, MacQueen and Siegel (1989) reported that an especially effective CS for demonstrating antisuppressive CRs was pentobarbital (Pent). That is, animals that received Pent-Cy pairings subsequently displayed substantial antisuppressive CRs in response to the barbiturate. This is in agreement with Revusky's (1985) suggestion that drug states in general, and the state induced by Pent in particular, may be especially appropriate CSs in studies of Cy conditioning. In the present experiment, Pent was used as the CS.

METHOD

Design

During the conditioning phase of the experiment, mice were injected with a subanesthetizing dose of Pent once a week for 3 weeks. This barbiturate was the CS. Groups differed with respect to the relationship between this Pent injection and injection of Cy. Mice in the paired group were injected with Cy 20 min after each Pent injection. Mice in the unpaired group were injected with Cy 24 h after each Pent injection. Finally, mice in the CS-alone group never received Cy; instead, they were injected with physiological saline 20 min after each Pent injection.

Following the conditioning phase, all mice were challenged with SRBC and reexposed to Pent on two occasions. Blood samples were then assessed for hemagglutinating activity. This initial assay constituted the preextinction assessment. The preextinction assessment provided an opportunity to evaluate the effects of conditioning on immunological activity in an experiment similar in design to other Cy conditioning experiments (see MacQueen & Siegel, 1989). On the basis of results of earlier work, it would be expected that the group with no preassay history of Cy (CS alone) would display normal immunoreactivity to the SRBC challenge, and the group with a history of Cy, but not of Cy paired with Pent (unpaired), should display immunosuppression as a result of the residual effects of Cy. The immunological CR is revealed in antibody titer levels of paired-group mice.

The extinction phase of the experiment started after preextinction assessment of antibody activity. Each of the three groups of mice was further divided into two subgroups, extinction and rest. Extinction subgroups received seven additional injections of Pent (one injection every 3 days). Rest subgroups were not injected during this period. Finally, all mice were again injected with SRBCs and reexposed to Pent, and antibody titers were again determined with a hemagglutination assay. This second determination of antibody activity (which was a measure of secondary antibody response) constituted the postextinction assessment. The postextinction SRBC challenge occurred 47 days after the last injection of Cy. Comparison of hemagglutination activity of paired-rest, unpaired-rest, and CS-alone-rest mice revealed the effects of conditioning manipulations long after the injection of Cy, when the drug's immunosuppressive effect would presumably no longer be manifest. Evalua-

tion of hemagglutination activity of the extinction subgroups of the three experimental groups revealed the effects of repeated presentation of the CS on any conditional immunomodulation apparent in the rest subgroups.

The experiment was conducted in two replications. The replications were conducted 10 months apart by different experimenters.

Procedure

The procedures for the first replication of the experiment are described below. With the exceptions noted, the second replication was conducted in an identical manner.

Subjects and drugs. Wildstrain male mice, bred at McMaster University, were group housed in plastic cages (4–6 per cage). They had free access to food and water throughout the experiment and were maintained on a 10-h light cycle (lights on at 11:00 p.m. and off at 9:00 a.m.). The mice ranged in weight from 11 to 31 g at the start of the experiment (25–36 g in Replication 2). They were randomly assigned to paired, unpaired, and CS-alone groups. (In Replication 1, 17 mice were assigned to the paired group, and 20 mice to each of the other two groups. In Replication 2, 20 mice were assigned to each group.)

All injections were i.p. Pentobarbital was injected at a dose of 20 mg/kg, in a volume of .615 ml/kg. Cyclophosphamide was injected at a dose of 100 mg/kg (in a volume of 10 ml/kg). Physiological saline injections were equated volumetrically with Cy injections.

Conditioning. Subjects received three conditioning trials, with a 7-day interval between trials. Conditioning trials occurred between 11:00 a.m. and 1:00 p.m. for animals in Replication 1, and between 3:30 p.m. and 5:30 p.m. for animals in Replication 2. Each subject was injected twice on each trial. For subjects in the paired and unpaired groups, the first injection consisted of Pent, and the second of Cy. For paired-group mice, the Pent–Cy interval was 20 min. For unpaired-group mice, this interval was 24 h. Unpaired-group mice were injected with Pent on the day before paired-group mice, so that both were injected with Cy on the same day. Mice in the CS-alone group were treated like mice in the paired group, except that the second injection consisted of saline, rather than Cy.

After the third conditioning trial, all mice received a 14-day recovery period. Immediately after the recovery period, antibody response was stimulated by injecting all mice with SRBCs (.6 ml of a 1% solution, approximately 1.5×10^8 cells/ml). Thirty minutes after administration of this antigen (20 min in Replication 2), all mice were injected with Pent. Thus, in accordance with the procedure of others (see Ader & Cohen, 1975; MacQueen & Siegel, 1989), mice were again presented with the CS on the day of the antigenic challenge. This constituted the first reexposure day. Three days later, mice received their second reexposure day (i.e., they were again injected with Pent).

Preextinction assay. Three days after the second reexposure, mice were anesthetized (Pent, 65 mg/kg), and cardiac punctures were performed to obtain approximately .35 ml blood from each mouse. The blood was centrifuged, and the serum was heat-inactivated at 56°C for 30 min. Antibody titrations were performed, using the technique described by Ader and Cohen (1975). Samples were assessed approximately 4 h after the titrations were performed. Titers were recorded as $\log_2$ reciprocals of the endpoint dilutions.

Extinction. The extinction phase of the experiment started 6 days after blood was obtained for the preextinction assay. Although some animals did not recover from the cardiac puncture, those that did survive appeared to be fully active at this time. Surviving animals were randomly assigned to either rest or extinction subgroups. For Replication 1, there were 7 mice in each subgroup during the extinction phase, except for unpaired–rest, which had 8 mice. In Replication 2, the number of subjects in each subgroup was as follows:

paired–rest, 7; paired–extinction, 8; CS-alone–rest, 9; CS-alone–extinction, 10; unpaired–rest, 9; unpaired–extinction, 9.

Extinction mice received seven further injections of Pent. In Replication 1, but not Replication 2, each Pent injection was followed 20 min later by injection of saline. These extinction trials occurred at 3-day intervals. Rest mice were left undisturbed during this period.

Four days after the last extinction-phase Pent injection, all mice were challenged with another SRBC injection, and were reexposed to Pent. Three days later, all mice received a final reexposure to Pent.

Postextinction assay. Two days after the final Pent reexposure (3 days in Replication 2), the mice were anesthetized, blood was collected, and a second passive hemagglutination was performed. The procedures for this postextinction assay were the same as those for the preextinction assay.

RESULTS

Similar results were obtained in each replication. The results from each replication are presented separately to emphasize the consistency.

Preextinction Assay

The mean hemagglutination antibody titer values (± 1 SEM) obtained for each group on the preextinction assay (for both replications) are shown in Figure 1. A 3 (group) $\times$ 2 (replication) analysis of variance of the data summarized in Figure 1 indicated that the groups differed significantly [$F(2,111) = 20.9$, $p < .001$]. Subsequent pairwise comparisons (Tukey's tests) revealed that the unpaired group differed significantly from each of the other two groups (all $ps < .001$), but the difference between the paired and CS-alone groups was not significant. Neither the effect of replication nor any interaction involving this variable was significant.

Postextinction Assay

The mean hemagglutination antibody titer values (± 1 SEM) obtained for each group on the postextinction assay (for both replications) are shown in Figure 2. The immune response for the postextinction assay was initiated by a second administration of SRBCs. The secondary antibody response is characterized by titer levels higher than those for the initial antibody response (see Roitt, Brostoff, & Male, 1985). This is indicated in the present experiment by the higher titer levels displayed on the postextinction test as opposed to the preextinction test, which can be seen by comparison of Figure 2 with Figure 1. In fact, all 95 mice providing data for both tests displayed postextinction titer levels that were higher than preextinction titer levels.

As is indicated in Figure 2, in both replications paired–rest subjects displayed the highest titers, with the remaining groups displaying similar levels of antibody activity.

The data summarized in Figure 2 were subject to a 3 (conditioning treatment) $\times$ 2 (extinction treatment) $\times$ 2 (replication) analysis of variance. The results of this anal-

Figure 1. Mean preextinction hemagglutination antibody titers (± 1 SEM) for all groups in each replication.

ysis indicated that titer values were significantly higher in Replication 1 than in Replication 2 [$F(1,83) = 19.6$, $p < .001$], but the replication variable did not significantly interact with any other variable. There was a significant interaction between conditioning and extinction treatments [$F(2,83) = 15.2$, $p < .001$]. Subsequent pairwise comparisons revealed the source of the interaction: paired-rest subjects, compared with subjects in all other groups, evidenced immunoenhancement. This finding was statistically significant in each replication (all $ps < .01$).

DISCUSSION

Preextinction Test

The results of the preextinction test in this experiment with mice are consistent with those reported by MacQueen and Siegel (1989) with rats. Unpaired-group mice, compared with CS-alone mice, displayed immunosuppression. That is, animals with a history of three Cy administrations display residual immunosuppression in response to an SRBC challenge 14 days after the last injection of the immunosuppressant. This persistent immunosuppression was reversed in paired-group mice; the usual effect of Cy in compromising immunological functioning was attenuated or eliminated by reexposure to the pharmacological CS previously paired with Cy. These preextinction test results are also similar to those reported by others with nonpharmacological CSs (Gorczynski et al., 1984; Krank & MacQueen, 1988).

Postextinction Test

The postextinction tests provided an opportunity to evaluate immunological conditioning in animals receiving

an antigenic challenge 47 days after the last Cy administration. In the rest-subgroup mice, which were left undisturbed during the extinction phase, no residual suppressive effect of Cy was evidenced. That is, unpaired-rest mice (which received Cy during the conditioning phase) displayed antibody titers similar to those seen in CS-alone-rest mice (which never received Cy). However, even long after the last conditioning trials, the Pent-Cy association was still apparent. Paired-rest mice displayed clear immunoenhancement; they responded to the Pent cue with significantly higher antibody titers than did unpaired-rest or CS-alone-rest mice. The results from these rest subgroups indicated that the effects of Pent-Cy pairings, in common with other conditional responses (Mackintosh, 1974), display substantial retention. Moreover, the pairings establish a conditional immunoenhancement. In previous experiments (and in the preextinction test of the present experiment), in which there was a shorter interval between training with Cy and assay of immunological activity, the effects seen in the paired group were compared with those seen in groups suffering residual Cy-induced immunosuppression. In such experiments, the compensatory immunological CR was manifest as an attenuation of the immunosuppressive effect of Cy. The results of the postextinction test in the present experiment indicate that animals can actually learn to augment immunological processes in response to a cue paired with an immunosuppressive drug.

The performance of the extinction subgroups on the postextinction test further indicate the associative basis of the CS-elicited immunoenhancement. Paired-extinction mice, which were exposed to Pent on seven occasions prior to postextinction assessment, no longer dis-

Figure 2. Mean postextinction hemagglutination antibody titers (+1 SEM) for rest and extinction conditions of all groups in each replication.

played immunoenhancement in response to Pent. Thus, although the factors that favor the development of an immunoenhancement CR are unclear, such a CR (in common with an immunosuppressive CR; see Bovbjerg et al., 1984) can be extinguished.

CONCLUSIONS

The present results indicate that Pent, following pairing with Cy, elicits immunoenhancement. This CR is extinguished by repeated Pent administrations. Observation of this compensatory CR is clearest if the conditional immunological effect is evaluated a considerable period of time after the last Cy administration, so that subjects are no longer experiencing residual immunosuppression.

Although these findings are consistent with those of others who have reported antisuppressive CRs following training with Cy, they contrast with reports of conditional immunosuppression. The reasons for the various outcomes are not yet clear. Perhaps CS modality (Krank, 1989; Krank & MacQueen, 1988), or circadian and exogenous stress factors (Gorczynski et al., 1984) contribute to the expression of the various immunological CRs. In any event, CRs seen following training with immunosuppressive drugs, in common with CRs seen following training with a variety of other drugs, may be opposite in directions to the drug effect (see Siegel, 1989). Other evidence for compensatory conditioning of the effects of an immunomodulating drug has been presented by Dyck and colleagues (Dyck, Driedger, Nemeth, Osachuk, & Greenberg, 1987; Dyck, Greenberg, & Osachuk, 1986; Dyck,

Osachuk, & Greenberg, 1989). They suggest that the CR to the immunostimulatory drug, polyinosinic polycytidylic acid (poly I:C), is a compensatory immunosuppressive response.

As has been indicated by MacQueen and Siegel (1989), the fact that Pent-Cy associations are readily formed has implications for the design of treatment schedules for patients receiving immunosuppressants. Patients receiving Cy are sometimes pretreated with sedatives, such as pentobarbital. Concern about this practice has been aroused by demonstrations that barbiturates modify the pharmacodynamics of Cy via their effects on microsomal enzymes of the liver (Hoshi, Kanzawa, & Kureitani, 1969). However, inasmuch as this effect appears minimal (Jao, Jusko, & Cohen, 1972), pentobarbital is combined with Cy in clinical settings (Donelli, Colombo, & Garattini, 1973). It is possible that such a treatment regimen favors the development of a Pent-Cy association. The effects of such inadvertent cuing of the immunosuppressant should be considered in the design of treatment schedules for patients undergoing chemotherapy.

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NOTES

1. It may well be the case that organisms respond with actions that are generally adaptive, rather than with actions that are appropriate to artificial and unusual situations (created, for example, by injection of immunosuppressive drugs). Thus, the conditional immunosuppression (and conditional increase in mortality) reported by Ader and Cohen (1975) was not "adaptive," but may have been an unfortunate by-product of processes that are generally beneficial (see MacQueen, MacRae, & Siegel, 1989). Indeed, there are circumstances in which conditional immunosuppression prolongs survival (Ader & Cohen, 1982).
2. In the experiments that have permitted evaluation of the effects of a Cy-paired CS long after conditioning, there were no direct measures of immune system functioning. Rather, conditional immunosuppression was inferred because reexposure to the CS had a salutary effect in mice suffering from a congenital autoimmune disorder (see, e.g., Ader, 1985).
3. Indeed, most demonstrations of antisuppressive CRs have used non-gustatory CSs, but there are some demonstrations of such compensatory conditioning with SAC as the CS (e.g., MacQueen & Siegel, 1989, Experiments 1-3). In such cases, Krank and MacQueen (1988) suggested, unauthorized environmental cues concurrently present with SAC overshadowed (Kamin, 1969) the gustatory cue.

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