Conditioned suppression of contact sensitivity is independent of sympathetic splenic innervation

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Exton, Michael S., Alexandra Elfers, Woo-Young Jeong, Diane F. Bull, Jürgen Westermann, and Manfred Schedlowski. Conditioned suppression of contact sensitivity is independent of sympathetic splenic innervation. Am J Physiol Regulatory Integrative Comp Physiol 279: R1310–R1315, 2000.—The present study investigated the role of sympathetic innervation of the spleen in conditioned suppression of a contact hypersensitivity (CHS) reaction. Behavioral conditioning was achieved by pairing saccharin drinking solution (conditioned stimulus, CS) with injection of cyclosporin A (CsA, 20 mg/kg; unconditioned stimulus, UCS). Four days after sensitization of the animals by application of a 5% 2,4-dinitrochlorobenzene (DNCB) to abdominal skin, the animals were challenged by applying a 1% DNCB solution to the ear. The CHS response was monitored by measuring the degree of ear swelling. Saccharin re-presentation reduced ear swelling to a magnitude that approached that achieved by CsA treatment. Histological examination demonstrated that the conditioned reduction of ear swelling was produced by a reduced leukocyte infiltration of the ear. Prior sympathetic denervation of the spleen did not alter the conditioned suppression of the CHS response. These data indicate that behavioral conditioning using CsA produces alterations of CHS that, unlike conditioned prolongation of heart allograft survival, are independent of sympathetically regulated conditioned alterations in the spleen.

Classical conditioning; cyclosporin

BEHAVIORAL OR CLASSICAL CONDITIONING is an associative learning paradigm, which pairs presentation of a benign novel stimulus (conditioned stimulus, CS) with a stimulus that produces physiological changes (unconditioned stimulus, UCS). Upon re-presentation of the CS, the organism produces physiological alterations that are usually ascribed to the UCS. This paradigm has been implemented to produce conditioned alterations in immune functions (1). Commonly, animals are presented with a sweet taste (saccharin) in the drinking water and subsequently injected with a pharmacological agent that produces changes in immune status. At a later date, the saccharin solution is re-presented, with the animals avoiding the stimulus (conditioned taste aversion) and experiencing concomitant alterations in immune function concordant with the effect of actual drug administration.

We established a model of behavioral conditioning that pairs saccharin as the CS with cyclosporin A (CsA) as the UCS (9, 10, 29). This paradigm produces conditioned reduction of splenocyte proliferation and cytokine [interleukin (IL)-2, interferon (IFN)-γ] synthesis in the spleen (9). These alterations are biologically relevant, as the paradigm prolongs survival time of heterotopic heart allografts (9, 10). Furthermore, the immunological and transplantation effects observed previously are mediated via sympathetic innervation of the spleen, as splenic denervation completely blocks the conditioned changes (9, 10).

Therefore, to investigate whether the behavioral conditioning paradigm using CsA as a UCS is generalizable to other cell-mediated immune responses, we examined the effects of conditioning on the course of the murine model of allergic contact dermatitis, contact hypersensitivity (CHS) (21, 23). Furthermore, to investigate whether conditioned changes of immune function using the present model generally function via sympathetic innervation of the spleen (9, 10), we examined the conditionability of CHS suppression in animals with a denervated spleen.

MATERIALS AND METHODS

Animals. Eighty experimentally naive male dark agouti (DA) rats (Harlan Laboratories, Borchum, Germany) weighing between 220 and 250 g were used. All rats were allowed to habituate for 3 wk before experimentation. Animals were individually housed in standard plastic-based laboratory cages (40 × 26 × 15-cm high) with a wire mesh lid. Cages were kept in an air-conditioned, sound-proofed holding room at an ambient temperature of 24.0 ± 0.5°C. The animals had access to standard lab chow and tap water ad libitum, except during the water-deprivation phase of the experiment. A 12:12-h light-dark cycle was maintained throughout the experiment, with lights off at 0700. This allowed stimuli presentation to be conducted during the dark (active) cycle of the animals. All conditioning procedures were completed under

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on each of the first 3 CS re-presentation days (CS1-CS3) they were in an identical manner to the sham-conditioned animals. However, water (Wat) instead of saccharin. CsA-treated rats were conditioned the conditioned animals, with the modification that they received water deprivation regimen for 5 days, allowing them 15 min of drinking at 0700 and again at 1700 each day (Fig. 1A). The present study implemented a three learning (CS-UCS pairing) trial paradigm. Each learning trial was separated by 72 h. This ensured that CsA was completely metabolized between learning trials. On the 5th day, animals received the first of three CS-UCS pairings. Twenty-four hours after the final morning CS-UCS pairing, animals were sensitized with the contact antigen 2,4-dinitrochlorobenzene (DNCB). Three days after the final pairing, the CS alone was presented during each drinking session. This was repeated for the subsequent 2 days. One hour after the third morning, CS re-presentation animals were challenged with the contact antigen. Ear swelling was measured 24 h later.

Animals were divided into conditioned, sham-conditioned, and CsA-treated groups (n = 10 per group; Fig. 1B). Conditioned animals received 0.2% saccharin solution as the CS, paired with 20 mg/kg ip CsA as the UCS on the training days. In the afternoon session they were administered water paired with intraperitoneal saline injection. Sham-conditioned rats were given water paired with CsA in the morning of the training days and saccharin in combination with saline in the afternoon. In addition, CsA-treated animals were treated similarly to sham-conditioned rats; however, these animals received an additional CsA injection (20 mg/kg) after each of the first three CS re-presentations. This allowed a comparison of the conditioned response with the actual drug effect.

Contact sensitivity. Contact sensitivity was induced by established methods (4, 17). Specifically, 200 μl of a 5% solution of DNCB (Sigma, St Louis, MO) dissolved in ethanol was applied to the shaven abdomen of the rat. Four days after sensitization, rats were challenged by the application of 20 μl of a 1% DNCB solution on each side of the right ear and 20 μl ethanol on each side of the left ear. Ear thickness was measured 24 h after challenge using a spring-loaded micrometer (Kroepelin, Schlüchtern, Germany) by an experimenter blind to the treatment groups. Ear swelling was recorded as the difference in ear thickness between the right and left ears and expressed in micrometers ± SE.

Splenic denervation. After the adaptation period, splenic denervation was conducted 2 wk before conditioning using standard techniques (9, 10). Briefly, a midline incision was used to open the abdominal cavity, exposing the splenic nerve vascular package. The splenic nerve bundle was isolated from the splenic vasculature, and the neural bundle was cut before its bifurcation. The incision was then sutured, and the animal was allowed to recover. Sham denervation was completed by again isolating the splenic nerve bundle without slicing the nerve.

Splenic catecholamine concentration. Denervation of the spleen was verified by measuring the level of splenic epinephrine and norepinephrine after measurement of ear swelling. Each spleen was homogenized in 0.1 M perchloric acid containing 0.1 mM EDTA. After centrifugation, 25 μl of the supernatant was used for catecholamine extraction (9). Norepinephrine concentrations were analyzed by HPLC (Gynotek, Germany) and expressed as nanograms per gram wet tissue. Intra-assay variability was <10%.

Histology. Both DNCB- and alcohol-treated ears were removed, frozen in liquid nitrogen, and stored at −70°C. Cryostat sections were made (thickness = 5 μm), air dried, wrapped in aluminium foil, and stored at −20°C. The slides were fixed for 10 min in equal parts of methanol and acetone (−20°C), washed in Tris-buffered saline containing 0.05% Tween 20, and incubated for 30 min at room temperature in a moist chamber with a mouse anti-rat monoclonal antibody to identify CD4+ and CD8+ T lymphocytes, B lymphocytes, monocytes, and natural killer cells (30). Then the slides were incubated with the second-step antibody (rabbit anti-mouse, Dako, Hamburg, Germany) and the third mouse antibody complex alkaline phosphatase anti-alkaline phosphatase (Dako) for 30 min. To enhance stain intensity, the second and third step antibody incubation steps were then repeated, each for 15 min duration. The positive cells were subsequently revealed in blue using Fast blue. After washing with Tris-buffered saline the slides were counterstained with hematoxylin (Fluka, Buchs, Switzerland) and mounted in glycergel (Dako).

Statistical analyses. Average differences in ear swelling between groups were analyzed using one-way ANOVA. Post hoc Fisher’s least significant difference tests were implemented to examine specific differences between groups. Values are presented as means ± SE. Statistically significant differences are reported when P < 0.05.
RESULTS

Behavioral conditioning suppresses CHS response. Conditioned animals in the present paradigm avoid consumption of the saccharin stimulus after the first CS-UCS pairing (conditioned taste aversion) (9, 10, 29). Such a response indicates acquisition of the association between the two stimuli and was observed in the present series of experiments, with conditioned animals typically drinking <10% of the level of saccharin consumed by control animals (data not shown).

Behavioral conditioning reduced DNCB-induced ear swelling compared with sham-conditioned animals. The magnitude of suppression approached that observed in rats administered a short-course therapeutic regimen of CsA (Fig. 2). Histological examination confirmed that the reduction of ear swelling in CsA-treated and conditioned animals was produced by an inhibition of leukocyte infiltration into the ear (Fig. 3).
No differences in specific leukocyte subset infiltration were observed (data not shown).

Conditioned suppression of CHS response is not mediated by sympathetic innervation of the spleen. Because we demonstrated that behaviorally conditioned prolongation of heart allograft survival is mediated via sympathetic innervation of the spleen (10), we examined the role of splenic innervation in suppression of the CHS response. Compared with sham-denervated rats, surgical denervation reduced splenic epinephrine and norepinephrine content to <20 and <10%, respectively (Fig. 4). Denervation per se had no effect on ear swelling, as no difference was observed between sham-conditioned animals that were either denervated or sham denervated. Additionally, surgical denervation of the spleen did not abrogate the behaviorally conditioned suppression of ear swelling (Fig. 5).

DISCUSSION

The behavioral conditioning paradigm used presently suppresses splenocyte proliferation and production of Th1 cytokines (IL-2 and IFN-γ) (9). Furthermore, these changes in cellular immune function are of sufficient magnitude to extend survival time of transplanted organs (9, 10). The current data demonstrate that conditioned changes are able to suppress the development of another T cell-mediated immune response, namely, contact sensitivity to haptens. However, in contrast to conditioned prolongation of heart allograft survival, the conditioned suppression of contact hypersensitivity is independent of sympathetic innervation of the spleen, as splenic denervation did not abrogate the conditioned effect.

The conditioned suppression of contact sensitivity using CsA as a UCS demonstrates that the conditioned effect is generalizable to other cell-mediated immune responses. Contact hypersensitivity is dependent on T cell IL-2, tumor necrosis factor (TNF)-α, and IFN-γ production (8, 31, 32). As we have shown that the present paradigm suppresses splenocyte IL-2 and IFN-γ production, it is likely that conditioning reduces contact sensitivity via inhibition of production of these cytokines.

We have shown that the functionally relevant suppression of cytokine production in the spleen is mediated via sympathetic innervation. Sympathetic nerves are in close contact with lymphocytes in the spleen (11, 19), and they release catecholamines that influence splenic IL-6, IL-2, and IFN-γ production (26) via functional adrenoceptors (18, 25). Therefore, we examined whether the conditioned suppression of IL-2 and IFN-γ production of splenocytes also contributes to development of conditioned reduction in contact sensitivity. Splenic denervation did not alter the conditioned suppression of contact sensitivity, indicating that conditioning alters immune changes via a number of simultaneous mechanisms.

The acute elicitation phase of contact sensitivity used presently is relatively independent of immune function of the spleen (12, 13, 16, 31). Specifically, challenge with the hapten results in CD4+ and CD8+ T cell infiltration of the draining lymph nodes; production of IFN-γ, IL-2, and TNF-α; recruitment of other inflammatory cells; and subsequent infiltration to the challenge site resulting in the characteristic edema (5, 8, 27, 31). Although the primary immune response to sensitization is partially regulated by the production of

Fig. 4. Compared with sham denervation, splenic denervation reduces concentration of epinephrine (A) and norepinephrine (B) in the spleen to <20 and <10%, respectively (n = 10 per group; ***P < 0.001 compared with denervated animals).

Fig. 5. Splenic denervation does not abrogate the conditioned reduction of contact sensitivity. Conditioned animals with a denervated spleen display suppressed contact sensitivity that mimics the effect observed in both conditioned rats that do not have a denervated spleen, as well as CsA-treated animals (n = 10 per group; *P < 0.05; **P < 0.01 compared with sham-conditioned animals).
**REFERENCES**


IFN-γ, IL-2, and IL-12 in the spleen (2, 20), the acute secondary immune response occurs primarily in draining lymph nodes, independent of splenic cytokine production (12, 13, 16, 31). Thus the present data show that conditioning has altered immune function specific to contact sensitivity inflammation.

The mechanisms whereby conditioning achieves a suppression of contact sensitivity are unclear. We have shown that conditioned alterations of cellular immunity in the spleen are mediated via sympathetic innervation (9, 10). As lymph nodes are also innervated by sympathetic nerves (11, 19), it is possible that conditioning may suppress the inflammatory response via neural innervation of the draining lymph nodes. In contrast, we cannot rule out the possibility that humoral mediators produce the current effects. High levels of corticosterone suppress, where mild stress-induced elevations of corticosterone enhance CHS after challenge (6, 7). Furthermore, as stress-induced alterations of CHS are abrogated by adrenalectomy (7), the current suppression of CHS may have been produced by adrenal hormones. However, as we have not been able to find any changes in corticosterone levels in response to the current paradigm, this seems unlikely. Nevertheless, it must be considered that other neuroendocrine factors may play a role in producing conditioned suppression of CHS (3, 14, 23, 28).

Conditioning produced a reduction of leukocyte infiltration of the challenged ear. This may have been produced by a number of mechanisms. First, conditioning may have suppressed the proliferation of T cells in lymph nodes, mimicking the effect of CsA (21, 23, 24). However, the swelling of cell numbers in draining lymph nodes during the CHS reaction is due primarily to cellular infiltration rather than proliferation (8, 27). Therefore, conditioning may have suppressed T cell production of IL-2, IFN-γ, and TNF-α in draining lymph nodes, thus reducing cellular infiltration to the challenged ear (5, 8, 31). Alternatively, conditioning may have suppressed chemokine-regulated recruitment of T cells from peripheral blood to the lymph nodes (15, 27). Thus examination of draining lymph node and peripheral blood cytokine profiles is required to help elucidate the mechanism of conditioned CHS suppression.

In summary, the current data show that behavioral conditioning can mimic the CsA-induced suppression of a CHS response. In contrast to prolongation of heart allograft survival, this effect is independent of sympathetic innervation of the spleen. This suggests that the current paradigm has the ability to reproduce changes in immune function that are predominantly not mediated by changes in the spleen. Nevertheless, it is presently unclear whether this is also produced by neural innervation of draining lymph nodes or via other endocrine factors in peripheral blood.

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