Relationship between cold tolerance and generation of suppressor macrophages during acute cold stress

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[1]*Department of Hygiene, National Defense Medical College, Tokorozawa 359;* [2]*Department of Health and Sports Sciences, University of Electro-Communications, Chofu 182;* [3]*Institute of Health and Sport Sciences, University of Tsukuba, Tsukuba 305, Japan; and* [4]*Laboratory of Exercise Physiology, University of Physical Education, 1124 Budapest, Hungary*

Kizaki, Takako, Tomomi Ookawara, Tetsuya Izawa, Junichi Nagasawa, Shukoh Haga, Zsolt Radák, and Hideki Ohno. Relationship between cold tolerance and generation of suppressor macrophages during acute cold stress. *J. Appl. Physiol.* 83(4): 1116–1122, 1997.—Acute cold stress induces suppressor macrophages expressing large numbers of receptors to the crystallizable fragment (Fc) portion of immunoglobulin G (MAC-1$^+$FcγRII/III^{bright} cells), resulting in the immunosuppression of splenocyte mitogenesis. The generation of MAC-1$^+$FcγRII/III^{bright} cells is mediated by the action of glucocorticoids (GCs) through the GC-receptor. In the present study, the generation of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cells was closely related to the decrease of rectal temperature during 3-day exposure to 5°C. We next investigated the effects of improved cold tolerance on the generation of MAC-1$^+$FcγRII/III^{bright} cells during acute cold stress. Mice were adapted to cold by exposure to 5°C for 3 wk (cold-acclimated mice) and then reexposed to 5°C for 3 h (acute cold stress) after living at 25°C for 24 h. The rectal temperature of cold-acclimated mice was not decreased by the acute cold stress. In addition, the proportion of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cell population from cold-acclimated mice was unaffected by the acute cold stress. The cold acclimation significantly attenuated the increases in serum corticosterone levels and the expression of the GC-receptor mRNA on peritoneal exudate cells in response to acute cold stress. These results suggest that the altered GC response to acute cold stress by the improvement of cold tolerance inhibits the generation of suppressor macrophages during acute cold stress.

glucocorticoid; immunosuppression; cold stress; cold acclimation; brown adipose tissue

STRESS, which is generally defined as a state of altered homeostasis resulting from an external or an internal challenge (i.e., a stressor), is characterized by activation of both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). The resulting neurochemical changes have been demonstrated to affect immune function both directly and indirectly (20). Indeed, environmental and psychological stresses reduce cell-mediated immunity, which may in turn lead to high susceptibility of infection and facilitation of tumor growth or metastasis (10, 17, 24). Although most studies have shown stress-induced immunosuppressive effects (21, 26), others have found no changes or even an enhancement of the immune response after stressor application (15). Such controversial results may be attributable not only to the stressor characteristics but also to complicated responses of the neuroendocrine system to stress exposure. For example, under prolonged or chronic stress, excessive actions of products of the HPA axis and SNS can lead to alterations in the physiological response to acute stress in many body systems (6). Therefore, depending on length of exposure, the nature of the stressor, and the timing of stress application, immune functions are suppressed, enhanced, or remain unchanged.

Environmental temperature is known to modify the immune capacity of a number of animal species. For instance, cold-exposed animals showed qualitative and quantitative differences in the response to infections from various microorganisms (9, 22). In our previous study (14), we showed that the proliferative responses to concanavalin A (Con A) in spleen cells from mice exposed to 5°C for 24 h (acute cold stress) were significantly lower than those from control mice and that the depressed Con A responses were attributable to the suppressive regulation by adherent cells (representative of cells of monocyte/macrophage lineage). Furthermore, we have recently demonstrated that acute cold stress markedly increases suppressor peritoneal macrophages, which strikingly suppress Con A responses of spleen cells from control mice by releasing nitric oxide (12, 13). The suppressor macrophages were characterized by the expression of large numbers of type II/III receptor to the crystalizable fragment (Fc) portion of immunoglobulin (Ig) G (MAC-1$^+$FcγRII/III^{bright} cells) (13).

Because environmental stressors, including cold stress, are prevalent and sometimes unavoidable, their harmful influence should be understood so that treatment modalities can be developed. Thus we were interested in the differences of physiological responses to acute cold stress between naive and cold-acclimated mice. The present study was undertaken to investigate whether the improved cold tolerance by exposure to chronic cold stress prevented the depression of immune responses by an acute cold stress and focused in particular on the generation of suppressor macrophages.

METHODS

Mice. Male C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan) at 8 wk of age. Animals were reared at 25°C (control mice) or at 5°C with a 12:12-h light-dark cycle (lights off at 7 PM). Food and water were available ad libitum. The animals were cared for in accordance with the "Guiding Principles for the Care and Use of Animals" approved by the Council of the Physiological Society of Japan and based on the

Declaration of Helsinki 1964. In some experiments, the mice were acclimated to cold by being exposed to an environmental temperature of 5°C for 3 wk (cold-acclimated mice). Cold-acclimated mice were kept at 25°C for 24 h and then reexposed to 5°C for 3 h. Rectal temperatures were measured by a thermistor thermometer inserted 4 cm into the rectum before and during the cold exposure. Immediately after the cold exposure, the mice were killed by cervical dislocation. The interscapular brown adipose tissue (BAT) was removed and dissected free from all recognizable white adipose tissue or other connective tissue and was then weighed.

Cell preparation and flow cytometry. Peritoneal exudate cells were induced in the mice by an injection of 1.5 ml ip of liquid paraffin (Wako Pure Chemical Industries, Osaka, Japan) because the number of resident macrophages in the peritoneal cavity was not enough for the planned experimental procedures. In all experiments (acute cold exposure, chronic cold exposure, and acute cold exposure after chronic cold exposure), liquid paraffin was injected 4 or 5 days before the mice were killed. Immediately after the acute or chronic cold exposure, the peritoneal exudate cells were harvested by sterile lavage. Flow cytometry analysis was carried out as described elsewhere (11) by using a fluorescence-activated cell sorter (FACStar PLUS; Becton Dickinson, Mountain View, CA). Before the immunofluorescence test, the peritoneal cells (1×10^6) were incubated with mouse Ig in phosphate-buffered saline at 5°C for 30 min to reduce nonspecific binding to the Fc receptor. Thereafter, peritoneal exudate cells were treated with anti-FcγRII/III monoclonal antibody (MAb) (2.4G2, rat IgG2b, American Type Culture Collection, Rockville, MD) and fluorescein isothiocyanate-labeled goat anti-rat Ig antibody (Caltag Laboratories, South San Francisco, CA). Rat myeloma protein (rat IgG2b, Serotec, Oxford, England) was used as isotypic control antibodies. After extensive washing, the cells were treated with phycoerythrin (PE)-conjugated anti-MAC-1 MAb (Caltag Laboratories). After each step, the cells were extensively washed with phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO) and 0.1% NaN_3 to reduce nonspecific staining.

Measurement of serum corticosterone. Blood samples were obtained by decapitation of the mice immediately after the cold exposure. Blood was allowed to clot for 1 h, and serum was harvested after centrifugation. Serum corticosterone concentrations were determined by radioimmunoassay (Amersham Life Sciences, Arlington, IL). Serum was diluted 1:5 with borate buffer (0.02 M, pH 7.4) and incubated at 60°C for 30 min to denature corticosterone-binding proteins before the assay.

Reverse transcription-polymerase chain reaction (PCR). Total cellular RNA was extracted by the guanidinium-isothiocyanate method from peritoneal exudate cells or sorted cells from peritoneal exudate cells. Single-strand cDNA was synthesized with reverse transcriptase from 1 μg RNA and was used for PCR. Primer sequences were described previously (13). cDNAs were amplified by the PCR method under the following conditions: at 94°C for 1 min, at 55°C for 1.5 min, and at 72°C for 1.5 min with 26 cycles for β-actin or with 30 cycles for glucocortisoid (GC) receptor. PCR products were separated by electrophoresis on 4% acrylamide gel and were visualized by ultraviolet illumination after being stained with ethidium bromide.

Statistical analysis. All data are expressed as means ± SE. When only two means were compared, Student's *t*-test for unpaired samples was used. For more than two groups, the statistical significance of the data was assessed by analysis of variance. When significant differences were found, individual comparisons were made between groups by using the *t* statistic and adjusting the critical value according to the Bonferroni method (3). Differences were considered significant at $P < 0.05$.

RESULTS

Time course of rectal temperature and generation of MAC-1$^+$FcγRII/III^{bright} cells during cold stress. Figure 1 shows the effect of cold stress on the rectal temperature in naive mice. Within 1 h of cold stress, the rectal temperature was significantly decreased. The lowest rectal temperature was observed in mice at 24 h of cold exposure. Thereafter, the rectal temperature was increased and returned to its normal levels after 72 h of cold stress.

We then examined the effect of cold stress on the populations of peritoneal exudate cells. Figure 2*A* shows the data comparing typical profiles of two-color immunofluorescence staining with MAbs specific to MAC-1 and FcγRII/III on peritoneal cells from three to five mice each. Two distinct cell populations (MAC-1$^+$FcγRII/III^{bright} cells and MAC-1$^+$FcγRII/III^{dull} cells) could be seen in control mice and in mice exposed to the different periods of cold stress. It is apparent from Fig. 2*B* that the MAC-1$^+$ cells bearing higher amounts of the FcγRII/III molecule increased significantly in the peritoneal exudate cells from mice cond stressed for 3 h. The proportion of MAC-1$^+$FcγRII/III^{bright} cells was markedly increased in peritoneal exudate cells at 24 h of cold stress and decreased thereafter. As summarized in Fig. 2*C*, the highest proportion of MAC-1$^+$FcγRII/III^{bright} cells was demonstrated in peritoneal exudate cells at 24-h cold stress. These results suggested that the highest expression of FcγRII/III on the peritoneal exudate cells was induced when the effect of low temperature reached its peak.

BAT weight and cold-tolerance test. The time course of BAT weight in terms of weight per unit body weight is illustrated in Fig. 3. The significant increase of the

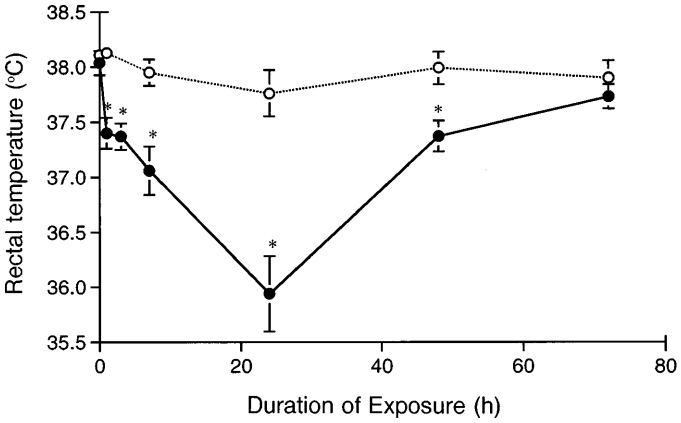

Fig. 1. Time course of rectal temperature of naive mice exposed to 5°C. Mice were kept at 25°C (○, $n = 8$) or 5°C (●, $n = 7$). Rectal temperatures were measured by thermistor thermometer inserted 4 cm into rectum at indicated time. Results are expressed as means ± SE. *Significantly lower than control value at same duration of exposure, $P < 0.01$.

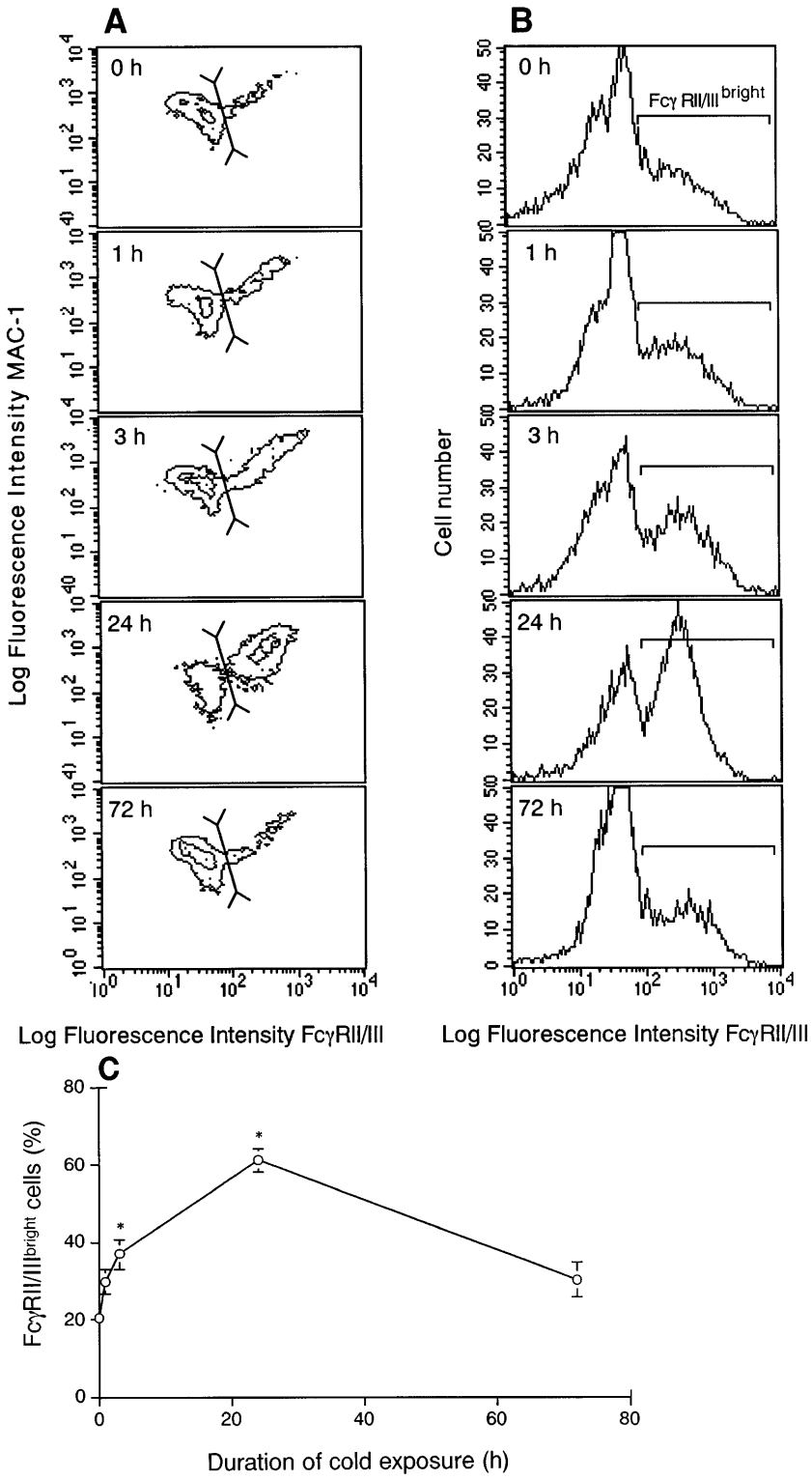

Fig. 2. Alteration of peritoneal exudate cell populations from unacclimated mice ($n = 4$ or 5) at different periods of cold exposure. Mice received an injection of liquid paraffin and were exposed to 5°C for indicated period (0–72 h). Peritoneal exudate cells were harvested at the same time. Expression of macrophages MAC-1 and FcγRII/III in peritoneal exudate cells was analyzed by flow cytometry. Cells (1×10^6) were stained with anti-FcγRII/III monoclonal antibody (MAb) followed by fluorescein isothiocyanate (FITC)-anti-rat immunoglobulin (Ig), then reacted with phycoerythrin (PE)-anti-MAC-1 MAb. *A*: two-color flow cytometric analysis of MAC-1 and FcγRII/III on peritoneal exudate cells. *B*: single histogram of FcγRII/III on MAC-1$^+$ peritoneal exudate cells. *C*: %FcγRII/III^{bright} cells (mean ± SE) in peritoneal exudate cells from 4 or 5 mice. *Significantly higher than 0-h value; $P < 0.01$.

BAT weight was observed in mice on *day 3* of cold exposure. The BAT weight of cold-acclimated mice was markedly higher than that of control mice, suggesting the improvement of cold tolerance. Cold tolerance of the cold-acclimated mice to an acute cold stress was then examined. The cold-acclimated mice were reared at 25°C for 24 h before the reexposure to acute cold stress (5°C for 3 h). Figure 4 shows the effect of acute cold stress on the rectal temperature in control and cold-acclimated mice. The rectal temperature in control mice decreased significantly during 3 h of acute cold stress, whereas the cold stress did not affect the rectal temperature of cold-acclimated mice. The finding suggested that the increased capacity of BAT developed by

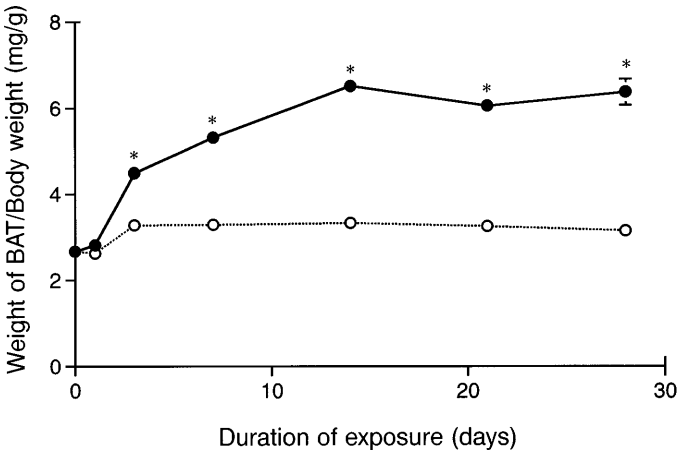

Fig. 3. Time course of brown adipose tissue (BAT) weight from mice exposed to 5°C. Mice were kept at 25°C (○, $n = 3$) or 5°C (●, $n = 4$) and killed after indicated period of cold exposure. Interscapular BAT was removed and weighed. Results are expressed as means ± SE. Error bars are too small to be distinguishable in the figure. *Significantly higher than control value at same duration of exposure, $P < 0.01$.

cold exposure for 3 wk is sufficient to maintain the rectal temperature in response to 3 h of cold stress.

Effects of cold acclimation on the generation of MAC-1$^+$FcγRII/III^{bright} cells by acute cold stress. To investigate the effects of the improvement of cold tolerance on the immune system, we investigated the generation of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cells by acute cold stress in cold-acclimated mice. As illustrated in Fig. 5*A*, before the acute cold exposure, the peritoneal exudate cell populations of cold-acclimated mice appeared to be almost the same as those of control mice. The proportion of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cells from control mice was significantly increased by 3 h of cold stress (Fig. 5, *A* and *B*). The proportion of MAC-1$^+$FcγRII/III^{bright} cells, on the other hand, did not increase in cold-acclimated mice with exposure to 3 h of cold stress. These findings are more clearly demonstrated in Fig. 5.

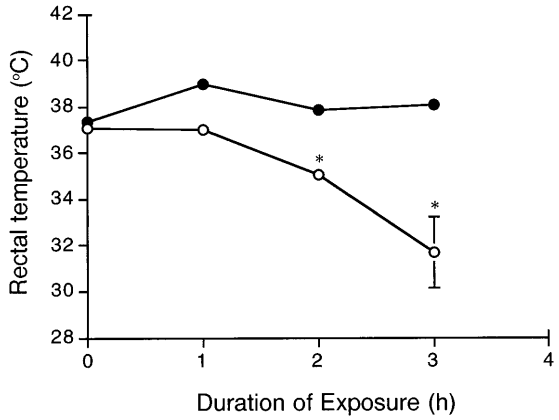

Fig. 4. Cold tolerance test on control and cold-acclimated mice. Control (○, $n = 5$) and cold-acclimated (●, $n = 5$) mice were exposed to 5°C. Rectal temperatures were measured by thermistor thermometer inserted 4 cm into rectum at indicated time. Results are expressed as means ± SE. Error bars are too small to be distinguishable in the figure. *Significantly lower than 0 h value, $P < 0.01$.

Effects of cold acclimation on GC responses to acute cold stress. As illustrated in Fig. 6, basal serum corticosterone concentrations of cold-acclimated mice were almost the same as those of control mice. Serum corticosterone concentrations of control mice increased markedly during 3 h of acute cold stress. On the other hand, the acute cold stress did not affect serum corticosterone concentrations of cold-acclimated mice.

Effects of cold acclimation on GC-receptor mRNA expression on peritoneal exudate cells by acute cold stress. GC-receptor mRNA was not detected in whole peritoneal cells from control mice but was detected in those from acute cold-stressed mice (Fig. 7). The expression of GC-receptor mRNA was observed in peritoneal exudate cells from cold-acclimated mice, but the expression was lower than in those cells from acute cold-stressed control mice. Although the expression level of β-actin mRNA was almost the same among all samples analyzed, the expression of GC-receptor mRNA in peritoneal exudate cells was unaffected by acute cold stress in cold-acclimatized mice.

DISCUSSION

Cold exposure stimulates heat production by means of nonshivering as well as shivering thermogenesis. A number of studies have now established that metabolic acclimation to cold is characterized by an enhanced nonshivering thermogenesis as a more efficient means of heat acquisition than shivering. BAT is a principal energy source of nonshivering thermogenesis through the presence of a tissue-specific uncoupling protein that is located in the inner mitochondrial membrane. During chronic exposure to cold, BAT mass increases, and this in turn enhances nonshivering thermogenesis (25). In the present study, the BAT mass increased significantly in mice exposed to cold for 3 days compared with the BAT mass in mice reared at 25°C. Meanwhile, the rectal temperature of naive mice exposed to cold was decreased during the initial 24 h, increased thereafter, and was completely restored to the normal temperature on *day 3* of cold exposure. Thus the increased mass of BAT on *day 3* of cold exposure was accompanied by an increase in thermogenesis. This increase in BAT mass may contribute to the restoration of the rectal temperature during acute cold stress.

In our previous study (13), we demonstrated that MAC-1$^+$FcγRII^{bright} cells suppressed Con A responses of spleen cells from control mice. This suggests that the acute cold stress generates suppressor macrophages that may cause immune suppression. We also showed that the MAC-1$^+$FcγRII^{bright} cells were at functionally high levels (13). Thus it appears that peritoneal exudate cells are activated in some way after acute exposure to cold. The observation that activated macrophages (MAC-1$^+$FcγRII^{bright} cells) function as suppressor cells is congruent with the concept that activated macrophages are more suppressive than their resident or nonactivated counterparts (1, 2, 18, 19, 23). On the other hand, during the course of inflammatory reac-

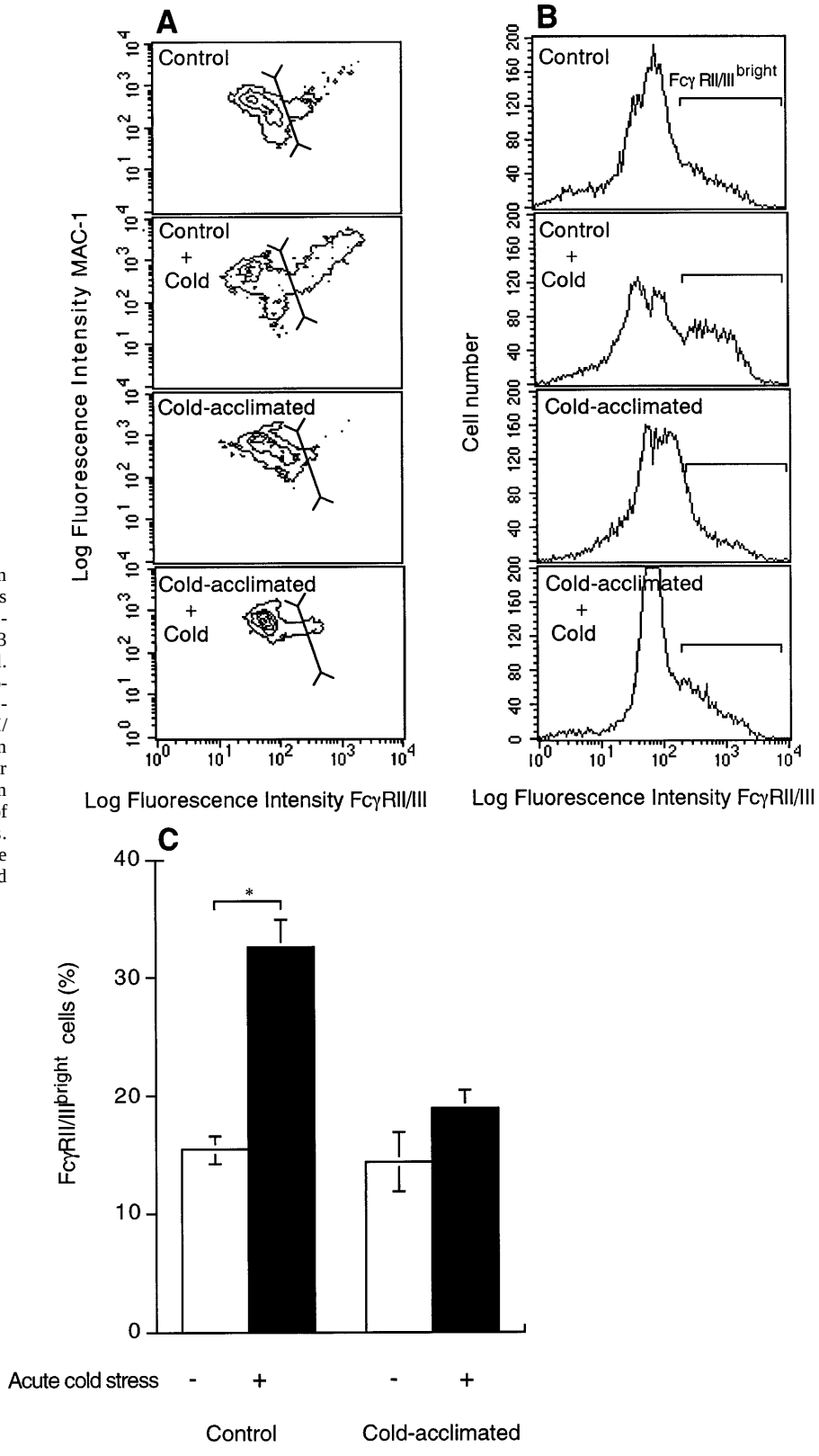

Fig. 5. Effects of cold acclimation on generation of FcγRII/III^{bright} cells in peritoneal exudate cells by acute cold stress. Control (n = 5) and cold-acclimated (n = 5) mice were exposed to 5°C for 3 h, and peritoneal exudate cells were harvested. Expression of MAC-1 and FcγRII/III on peritoneal exudate cells was analyzed by flow cytometry. Cells (1×10^6) were stained with anti-FcγRII/III MAb followed by FITC-anti-rat Ig and then reacted with PE-anti-MAC-1 MAb. *A*: 2-color flow-cytometric analysis of MAC-1 and FcγRII on peritoneal exudate cells. *B*: single histogram of FcγRII/III on MAC-1$^+$ peritoneal exudate cells. *C*: mean %FcγRII^{bright} cells in peritoneal exudate cells with SE. *$P < 0.01$, stressed vs. unstressed controls.

tions induced by infectious processes or by autoimmune responses, activated macrophage-derived cytokines, such as interleukin (IL)-1, IL-6, and interferon-α, affect the brain by activating the HPA axis and inducing fever, slow-wave sleep, and decreased appetites (4, 7, 16). Therefore, cells in monocyte/macrophage lineage appear to have a profound effect on host resistance, survival, and a variety of physiological responses to infection. In addition, the recent study by Hofman and Hinton (8) indicated that the pyrogenic effects of IL-1 in

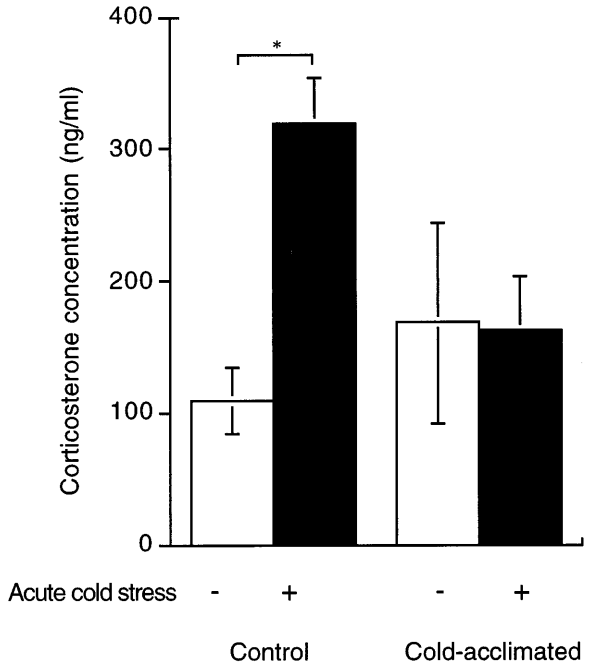

Fig. 6. Effects of cold acclimation on serum levels of corticosterone. Control ($n = 5$) and cold-acclimated ($n = 4$) mice were exposed to 5°C for 3 h, and serum levels of corticosterone were determined. $*P <$ 0.01, stressed vs. unstressed controls.

rats are mediated centrally and are caused by the sympathetic activation of thermogenesis in BAT, thereby resulting in a rise in the metabolic rate. Furthermore, Burysek et al. (5) have suggested a dual effect of IL-1 on BAT: one mediated centrally through sympathetic innervation and the other peripherally by direct interaction with adipocytes. As shown in Fig. 2, the proportion of MAC-1$^+$FcγRII^{bright} cells in peritoneal exudate cells

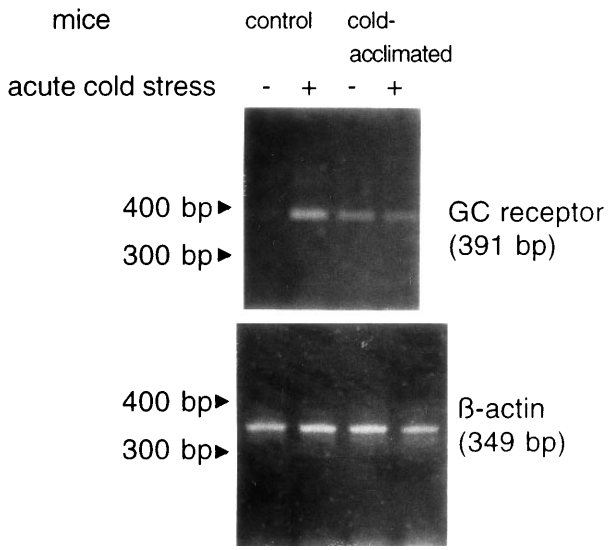

Fig. 7. Expression of glucocorticoids (GC) receptor mRNA on peritoneal exudate cells. Control and cold-acclimated mice were exposed to 5°C for 3 h. Expression of both GC-receptor and β-actin mRNA in peritoneal exudate cells from control mice, acute cold-stressed control mice, cold-acclimated mice, and cold-acclimated, acute cold-stressed mice were analyzed by reverse transcription-polymerase chain reaction. bp, Base pairs.

rapidly increased at an early stage of cold exposure when the rectal temperature was lowered, and the proportion decreased on *day 3* when normothermia was achieved. Although any conclusion is speculative, it seems that neuroendocrine pathways activated by the response to cold stress may result in macrophage activation and cytokine production that may result in thermogenesis. Our data could be interpreted to suggest that activated macrophages are important in early hyperthermia, whereas hyperthermia after *day 3* may be mediated by increased BAT mass.

In any event, the generation of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cells by acute cold stress appeared to be closely related to the decrease of rectal temperature. Using cold-acclimated mice, we then investigated whether improved cold tolerance would inhibit the immunomodulation by acute cold stress. BAT mass was greatly increased during chronic exposure to cold. The acute cold stress test clearly demonstrated improved cold tolerance in cold-acclimated mice. When cold-acclimated mice were reexposed to 3 h of cold stress after living at 25°C for 24 h, the rectal temperature did not change substantially, whereas the rectal temperature of control mice decreased significantly. The increased mass of BAT obtained by exposure to cold for 3 wk seemed to be sufficient to keep the rectal temperature constant. Cold acclimation did not affect the basal percentage of FcγRII/III$^+$ macrophages compared with nonacclimated mice. The proportion of MAC-1$^+$FcγRII/III^{bright} cells in peritoneal exudate cells from cold-acclimated mice, unlike those in unacclimated controls, was unaffected by 3 h of acute cold stress. Thus these results suggest that improved cold tolerance inhibits not only the decrease of body temperature but also the generation of suppressor macrophages during acute cold stress.

As already stated, stress, which is broadly defined as the response of an organism to stimulation or change, is characterized by activation of both the autonomic nervous system and the HPA axis. In our previous study, we demonstrated that the generation of the MAC-1$^+$FcγRII/III^{bright} cells during acute cold stress is mediated to a greater or lesser degree by increased GC levels after the activation of the HPA axis (13). Thus we investigated effects of acute cold stress on serum corticosterone concentrations of cold-acclimated mice. Cold acclimation did not affect basal corticosterone levels but did attenuate the corticosterone response to acute cold stress compared with nonacclimated controls. In addition, cold acclimation attenuated the increase in the GC mRNA expression in peritoneal exudate cells caused by acute cold stress. These observations, coupled with our previous findings that adrenalectomy and administration of the GC antagonist RU-38486 can block suppressor macrophage generation in unacclimated, acute cold-stressed mice (13), suggest that attenuated GC responses to acute cold stress in cold-acclimated mice may be the mechanism responsible for the lack of MAC-1$^+$FcγRII/III^{bright} cell generation during acute cold stress. The critical mechanisms that regulate the cellular immune responses characterized

in the present report and the role of FcγRII/III macrophages remain to be elucidated. Further studies will be needed to clarify the immunological or nonimmunological roles of cells of monocyte or macrophage lineage in the response to acute cold stress.

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