

## Chronic stress alters the immune response to influenza virus vaccine in older adults

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Communicated by John C. Liebeskind, University of California, Los Angeles, CA, December 28, 1995 (received for review October 24, 1995)

**ABSTRACT** To determine whether a chronic stressor (caregiving for a spouse with a progressive dementia) is associated with an impaired immune response to influenza virus vaccination, we compared 32 caregivers' vaccine responses with those of 32 sex-, age-, and socioeconomically matched control subjects. Caregivers showed a poorer antibody response following vaccination relative to control subjects as assessed by two independent methods, ELISA and hemagglutination inhibition. Caregivers also had lower levels of *in vitro* virus-specific-induced interleukin 2 levels and interleukin 1 $\beta$ ; interleukin 6 did not differ between groups. These data demonstrate that down-regulation of the immune response to influenza virus vaccination is associated with a chronic stressor in the elderly. These results could have implications for vulnerability to infection among older adults.

Caregiving for a spouse with progressive dementia can be stressful; caregivers must cope with severe behavioral problems including incontinence, the inability to communicate or recognize familiar people, and wandering (1). Dementia family caregivers typically develop anxiety and depressive symptomatology associated with caregiving, even in the absence of a prior mood disorder (2). The progressive dementias are not rapidly lethal, leading to potentially long periods of caregiving. For example, modal survival time after the onset of Alzheimer disease is up to 10 years, making caregiving a long-term or chronic stressor (3).

In addition to caregivers' increased vulnerability to depression, data from several laboratories suggest that caregivers have poorer immune function than matched control subjects, including reduced proliferative responses to mitogens and less enhancement of natural killer cell lysis in response to the stimulatory effects of recombinant interferon  $\gamma$  and recombinant interleukin 2 (IL-2) (4, 5). McCann (6) showed that delayed hypersensitivity skin testing was markedly poorer in 34 spousal caregivers than 33 comparable noncaregivers. In fact, compared to normal age and gender standards, 50% of the caregivers were totally or relatively anergic, compared to only 12% of noncaregiver controls. Consistent with these differences, caregivers reported significantly more days ill than controls, primarily from upper respiratory tract symptoms as independently confirmed by their physicians (4).

The immunological decrements associated with the stress of caregiving are of particular concern because older individuals already have age-related reductions in cellular immune function with important health consequences; respiratory infections such as influenza and pneumonia remain major causes of morbidity and mortality among older adults, and many older individuals do not respond to influenza virus vaccines (or other novel antigens) as efficiently as younger adults (7–11). Adults

who show poorer responses to vaccines and other antigenic challenges also experience higher rates of clinical illness, including influenza virus infection (10–12). Thus, we hypothesized that immunological responses to influenza virus vaccination would be poorer in spousal caregivers than in matched noncaregiving control subjects.

### MATERIALS AND METHODS

**Subject Recruitment Procedures and Screening Criteria.** Spousal caregivers were recruited from three local dementia evaluation centers in area hospitals, neurologists' referrals, the city's Alzheimer's Disease Association support groups and monthly newsletter, and respite care programs. While the majority of the caregivers' spouses had a diagnosis of Alzheimer disease ( $n = 21$ ), 2 were diagnosed as multiinfarct dementia, 7 as Parkinson disease with progressive dementia, and 2 with Huntington disease; comparisons of Alzheimer disease caregivers with caregivers for other progressive dementias suggest that dementia caregiving produces similar adverse effects in family members (1, 2). Caregivers spent a mean  $\pm$  SD of  $8.39 \pm 8.49$  hr per day in caregiving-related activities and reported they had been providing care for an average of  $7.25 \pm 3.46$  years.

Control subjects were recruited through newspaper advertisements, church groups, notices posted in senior citizen centers, and referrals from other participants; potential control subjects who reported any caregiving activities were excluded. Caregivers and control subjects were matched on sex, age, and family income; we used income as a proxy for socioeconomic status because many of our caregivers were older women who had not worked outside the home. Caregivers or controls with immunologically related health problems such as cancer or recent surgeries were excluded.

All subjects were part of a larger longitudinal project on stress and health in older adults (2, 4, 5). For this study, we used matched caregiver–control pairs who had all received an influenza vaccination in the previous year, excluding 22 subjects who had not been vaccinated, 23 former caregivers whose impaired spouse had died, and 36 controls for whom we had no matches among the current caregivers. The Ohio State University Biomedical Research Review Committee approved the project; all subjects gave written informed consent prior to participation.

**Subject Characteristics.** Each of the groups of 32 caregivers and 32 controls included 18 women and 14 men. Caregivers' mean  $\pm$  SD age was  $73.12 \pm 8.64$  years, compared to  $73.30 \pm 7.94$  years for controls, with a range of 53–89 years. The modal subject reported an annual family income between \$20,000 and

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Abbreviations: HA, hemagglutinin; HAI, hemagglutinin inhibition; IL, interleukin; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte.

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\$30,000 and had completed several years of college. The majority (93%) were Caucasian. Although most noncaregivers were married (23 of 32 or 72%), 7 were widowed, and 2 were divorced. While the groups were not matched on marital status, the inclusion of more divorced and widowed noncaregivers worked against confirmation of the experimental hypothesis: intact marriages are associated with lower rates of morbidity and mortality as well as better immune function (13, 14).

Importantly, all subjects had received an influenza virus vaccination in the prior year (Fluzone vaccine; Connaught Laboratories). Furthermore, 23 caregivers and 21 controls, and 19 caregivers and 17 controls had received the vaccine 2 and 3 years earlier, respectively. Thus, the groups had comparable vaccine histories (8, 15).

The Beck Depression Inventory (16) provided information on the severity of depressive symptoms. The 13 items on the short Beck cover affective, cognitive, and vegetative depressive symptoms. Subjects rate the severity of each symptom from 0 to 3.

We collected health-related data to assess the possibility that caregiving-immunological relationships might simply reflect the contribution of other variables. Plasma albumin levels and body mass data provided information on the nutritional status of subjects. Health questions from the Older Adults Resources Survey (17) assessed problems with lungs, kidneys, liver, digestive system, heart, high blood pressure, migraines, diabetes, hormonal conditions, thyroid, cancer, cataracts, teeth, hernia, gout, hardening of the arteries, circulatory system, prostate, ovarian or uterine, and muscle-related disorders.

**Vaccine, Timing of Samples, and Immunological Assays.** Fluzone vaccine was kindly provided by Connaught Laboratories for this study; the 1993–1994 trivalent Fluzone vaccine included A/Beijing/32/89, A/Texas/36/91, and B/Panama/45/90. The vaccine contained zonal purified, whole virus inactivated with formaldehyde. Fluzone vaccine was standardized to contain 45  $\mu\text{g}$  of hemagglutinin (HA) per 0.5-ml dose (15  $\mu\text{g}$  of HA per virus).

The first blood sample was obtained just prior to vaccination and a second was obtained 25–35 days after vaccination. Because some older individuals respond later than 4 weeks after vaccination (18), an additional blood sample was collected 6 weeks after inoculation from those individuals who had failed to show 4-fold antibody increase at 25–35 days.

Only those subjects who had shown a 4-fold antibody response to the vaccine as measured by ELISA were studied further at 3 and 6 months. This selection of responders for the 3- and 6-month blood draws was designed to assess those subjects who were most likely to show a concomitant influenza virus-specific T-cell response (i.e., an IL-2 response). Because the IL-1 $\beta$  and IL-6 mononuclear cell responses to lipopolysaccharide (LPS) stimulation should be independent of the response to vaccination (but important measures of cellular immunity), these responses were measured at only 2 time points (before and 25–35 days after vaccination). All blood samples were drawn between 8:00 a.m. and 11:00 a.m. to control for diurnal variation.

**ELISA.** Serum was collected from each blood draw and frozen at  $-70^{\circ}\text{C}$  until assayed for antibody titers. Antibody titers were determined using an ELISA for the complete Fluzone vaccine and each of the HA proteins (Beijing, Texas, and Panama). Briefly, 96-well plates were coated overnight at  $4^{\circ}\text{C}$  with Fluzone vaccine (0.9  $\mu\text{g}/\text{ml}$ ) or purified HA protein (0.3  $\mu\text{g}/\text{ml}$ ), washed, and incubated 2 hr at  $37^{\circ}\text{C}$ ; serum dilutions prepared in phosphate-buffered saline supplemented with 10% fetal bovine serum were then added. Plates were rewashed and goat anti-human IgG antibody and then alkaline phosphatase conjugate were added and plates were incubated 2 hr at  $37^{\circ}\text{C}$ . The secondary antibody was detected using Sigma

10 phosphatase substrate tablets (Sigma) in diethanolamine buffer and absorbance was read at 405 nm. Titers were determined by identifying the serum dilution at which the titration curve became asymptotic (19).

**HA Inhibition (HAI) Assay.** The HAI test was performed as described by the Centers for Disease Control (12, 20). Antigens were identical to those used for the ELISA and antibody titers were determined for all sera to each of the individual antigens. Chicken red blood cells were collected from a single chicken. Sera were treated with trypsin followed by periodate, glycerol, and sodium chloride. All sera were assayed in duplicate serial 1:2 dilutions from 1:10 to 1:2560 using reagents from a single batch, and serial sera collected from a given individual were tested on the same 96-well V-bottomed plate (Corning Costar, Cambridge, MA) for a given antigen. Titers were reported as the log average of the highest dilution at which hemagglutination was completely inhibited. Any duplicate serum titers differing by more than one dilution were retested with all sera for that individual to that antigen.

**Cytokine Assays.** Polyclonal stimulation with mitogen (LPS) was used to assess the proinflammatory cytokine responses (IL-1 $\beta$  and IL-6) of monocytes from caregivers and controls. Peripheral blood leukocytes (PBLs) were isolated by density gradient centrifugation on Ficoll-metrizoate gradients from 40 ml of heparinized venous blood. The isolated PBLs ( $2 \times 10^6$  cells per ml) were incubated in 1 ml of RPMI 1640 medium supplemented with 10% male human serum (Sigma) and stimulated with LPS or Fluzone vaccine (5  $\mu\text{g}/\text{ml}$ ) containing 11.25, 45.0, or 180 ng of HA protein per ml for 48 hr at  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2/95\%$  air. The Fluzone vaccine was first dialyzed for use as antigen. The cultures were then irradiated with 2000 rads and incubated at  $37^{\circ}\text{C}$  for another 24 hr at which time the supernatant was collected and frozen at  $-70^{\circ}\text{C}$  until assayed for cytokines.

Increased vulnerability to influenza infection among older adults is clearly associated with poorer cytokine responses, particularly the influenza virus-specific IL-2 response (8). IL-2 was assayed using the CTLL-20 cell bioassay (19) using concanavalin A- and Fluzone-stimulated culture supernates. LPS stimulated cultures were assayed for IL-1 $\beta$  and IL-6 by double-sandwich ELISA. The IL-1 $\beta$  ELISA used a commercially available monoclonal antibody (Genzyme) for coating the plates. Polyclonal rabbit anti-IL-1 $\beta$  antibody (Genzyme) was used as the primary antibody, and horseradish peroxidase-labeled goat anti-rabbit serum (Tago) was used as the detecting antibody. The IL-6 ELISA employed an anti-IL-6 monoclonal antibody (Biosource, Camarillo, CA) for coating the plates; a polyclonal goat anti-human primary antibody (Biosource) and horseradish peroxidase-labeled polyclonal swine anti-goat antibody were used (Tago). Both ELISAs used 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) as a substrate, with absorbance read at 405 nm. Recombinant human IL-1 $\beta$  and IL-6 (Biosource) were used as standards for each respective ELISA.

**Lymphocyte Subsets.** Monoclonal antibodies (Coulter) and flow cytometry were used to obtain percentages of T lymphocyte and monocyte populations as described (4). Mononuclear cells were obtained and prepared in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline. Approximately  $0.5\text{--}1.0 \times 10^6$  cells were aliquoted to  $12 \times 75\text{-mm}$  snap-cap tube(s) for reaction with the appropriate monoclonal antibodies: CD3 (total T lymphocytes), T4/T8 (helper/suppressor cells), MO2/KC56 (macrophage/monocytes). Percentages of lymphocyte populations were obtained by gating on lymphocytes; the percentage of macrophage/monocytes was obtained by gating on leukocytes. Flow cytometry was performed with a Coulter Epics Profile II flow cytometer.

**Statistical Methods.** Cytokine and antibody data were subjected to natural log transformations to normalize the distributions prior to analyses. We used univariate and multivariate

analyses of variance to assess differences between caregivers and controls, change over time, and the interaction of these variables; in addition, IL-2 analyses included an additional within-subjects variable, differences among the three Fluzone concentrations. The addition of age and income as covariates for cytokine analyses, a strategy that represents an additional level of rigor above the test for group differences on matching variables, did not change the results.  $\chi^2$  tests were used to analyze bivariate data.

Throughout our data, we found no significant gender differences. Thus, while gender was included as a variable in earlier analyses, sex differences will not be reported.

**RESULTS**

**Measures of the Immune Response to Influenza Virus Vaccination. Antibody responses.** A 4-fold antibody increase is the conventional standard for determining a significant response to viral vaccine (9, 11, 18). Thus, vaccine “responders” were defined as those individuals whose influenza antibody titers increased 4-fold or more to any one of the three individual vaccine components or to the total Fluzone vaccine when used as the antigen (i.e., all three HA components combined). Comparisons of prevaccination ELISA antibody titers showed no differences between caregivers and noncaregivers, all F values < 1.25 ( $P = 0.34$  for Beijing,  $P = 0.58$  for Panama,  $P = 0.27$  for Texas, and  $P = 0.29$  for total Fluzone vaccine as the test antigen).

Although caregivers and noncaregivers had comparable baseline antibody titers as measured by ELISA, caregivers responded less often after vaccination,  $\chi^2(1, N = 64) = 5.06, P = 0.02$ ; only 12 caregivers responded (38%), compared to 21 controls (66%), as shown in Fig. 1. These differences were magnified in older subjects: among those >70 years old, only 5 (26.3%) of the 19 caregivers responded, compared to 12 (60%) of the 20 controls,  $\chi^2(1, N = 39) = 4.50, P = 0.03$ ; among subjects <70 years old, 7 (53.8%) of the 13 caregivers responded, compared to 9 (75%) of the 12 controls,  $\chi^2(1, N = 25) = 1.21, P = 0.27$ . Two weeks later, an additional 5 caregivers and 2 controls had responded, bringing the totals to 17 (53%) and 23 (72%), respectively, to be studied further 3 and 6 months postvaccination. Thus, despite similar antibody titers prior to vaccination, caregivers responded less often after vaccination, with these group differences amplified in individuals over 70 years old.

HAI tests provided antibody data complimentary to the ELISA (Fig. 1); the ELISA has greater sensitivity, while the HAI has excellent specificity (21). As with the ELISA determinations, individuals who showed a 4-fold or greater HAI titer increase to any vaccine antigen were defined as “responders.” Comparisons of HAI prevaccination antibody titers again showed no differences between caregivers and noncaregivers

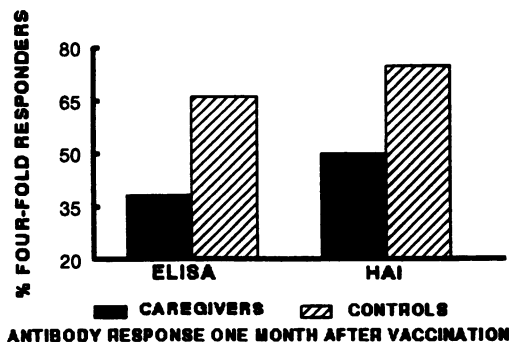


FIG. 1. Percentage of caregivers and controls showing a clinically significant four-fold increase in antibody 1 month after vaccination using antibody data from two different assays.

before vaccination, with all F values < 1 ( $P = 0.84$  for Beijing,  $P = 0.91$  for Panama, and  $P = 0.76$  for Texas). Caregivers were significantly less likely than controls to show a 4-fold increase in HAI antibody titers 4 weeks after vaccination,  $\chi^2(1, N = 64) = 4.27, P = 0.04$ ; 16 caregivers responded (50%), compared to 24 controls (75%). Thus, two independent methods for assessing antibody responses to vaccination demonstrated that caregivers showed a poorer response than noncaregivers.

**Cytokine responses.** IL-1 and IL-6 serve as amplifiers for T- and B-lymphocyte proliferation following challenge by a pathogen. As shown in Fig. 2, IL-1 $\beta$  was significantly lower in caregivers than controls,  $F_{(1,54)} = 13.08, P < 0.001$ . The change following vaccination was not significant,  $F_{(1,54)} = 3.46, P < 0.07$ , nor was the group by time interaction,  $F_{(1,54)} = 1.27, P < 0.27$ . In contrast, no significant differences were seen in the IL-6 responses, with F values < 1 for group ( $P < 0.35$ ), time ( $P < 0.92$ ), and the group by time interaction ( $P < 0.37$ ). *In vivo* studies have shown that chronic or long-term stress differentially affects cytokine responses, suppressing some but not others, with mixed data for IL-6 (22).

As illustrated in Fig. 3, caregivers produced significantly less IL-2 than controls,  $F_{(1,34)} = 4.70, P = 0.04$  (without a group by time interaction,  $F < 1, P = 0.60$ , or a group by concentration interaction,  $F < 1, P = 0.88$ ). The time effect for IL-2 was marginal,  $F_{(3,32)} = 2.77, P = 0.06$ , probably as a consequence of subjects' repeated influenza vaccinations in prior years. As noted earlier, only subjects who showed at least 4-fold antibody responses were followed and studied further at 3 and 6 months; thus, these group differences are especially noteworthy since all of these subjects had shown a significant antibody response to vaccination by 6 weeks. Notably, four of the five late-responding caregivers (i.e., those who showed a significant antibody response at 6 weeks but not at 4 weeks) did not produce detectable levels of IL-2, while the two later-responding controls both had measurable IL-2 responses after vaccination. McElhaney *et al.* (8) suggest that cytokine responses, particularly IL-2, may represent more important consequences of influenza vaccination among older adults than antibody changes.

**Percentages of T lymphocytes and monocytes.** The percentages of monocytes and CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> lymphocytes were used to assess the possibility that the differences in cytokine production simply reflected group differences in lymphocyte or monocyte percentages rather than real differences in cellular responses to stimulation. Caregivers and controls did not differ (all F values < 2.10) in percentages of monocytes ( $P = 0.98$ ), CD3<sup>+</sup> ( $P = 0.96$ ), CD4<sup>+</sup> ( $P = 0.56$ ), or CD8<sup>+</sup> lymphocytes ( $P = 0.16$ ). In prior work these lymphocyte subsets were not altered by an influenza vaccination (8).

**Psychological Data and Health-Related Behaviors.** Caregivers reported significantly higher levels of depressive symptoms on the short Beck Depression Scale than noncaregivers,

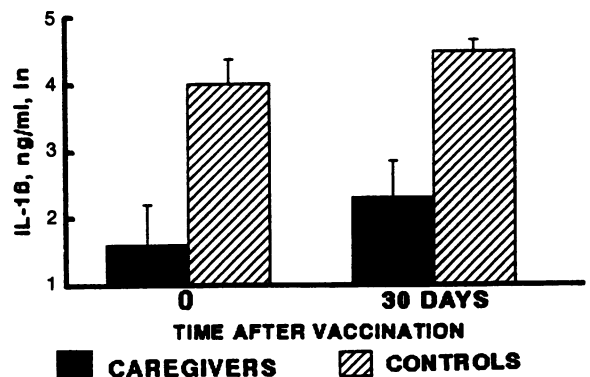


FIG. 2. IL-1 $\beta$  responses (mean  $\pm$  SEM) of caregivers and controls immediately prior to vaccination and 1 month after vaccination.

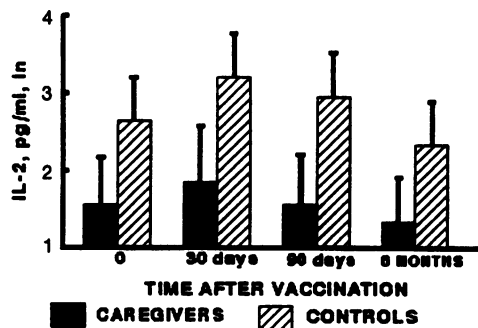


FIG. 3. *In vitro* IL-2 responses to Fluzone vaccine (mean  $\pm$  SEM) prior to vaccination and 1, 3, and 6 months after vaccination for those subjects who showed at least a 4-fold antibody increase. These data represent a specific T-cell response to the vaccine for the 11.25-ng/ml concentration of antigen.

$F_{(1,62)} = 12.46$ ,  $P < 0.001$ . Caregivers' mean  $\pm$  SD score was  $5.92 \pm 4.59$ , compared to noncaregivers' mean of  $2.69 \pm 2.24$ .

In accord with the majority of caregiving studies (1), caregivers' depressive symptoms were not reliably correlated with caregiving variables including years spent caregiving, the number of hours per day consumed by caregiving activities, or the extent of patient impairment. Similarly, immunological data were not significantly correlated with these variables in this relatively small sample. Inclusion of Beck Depression scores as a covariate in cytokine analyses did not alter the significance of group difference for IL-1 $\beta$  or IL-2, suggesting that the stressful effects of caregiving extend beyond depressive symptomatology, consistent with other research (4).

As noted earlier, nine controls (28%) were widowed or divorced, while all caregivers were married. However, marital status was not significantly related to controls' antibody or cytokine responses.

Health-related behaviors did not distinguish between caregivers and controls. Alcohol consumption was low and did not differ between groups,  $F < 1$ ,  $P = 0.72$ . Only one subject, a control, was a smoker. The two groups did not differ in body mass,  $F < 1$ ,  $P = 0.45$ , or weight change in the prior week,  $F_{(1,64)} = 2.18$ ,  $P = 0.14$ . All subjects had plasma albumin levels within the normal range (23).

Caregivers and controls did show reliable differences in sleep and exercise. Caregivers reported an average of  $22.44 \pm 2.44$  hr of sleep in the 3 days preceding vaccination, compared to controls'  $23.66 \pm 1.45$  hr,  $F_{(1,62)} = 5.87$ ,  $P = 0.02$ . However, correlations between sleep and immunological data were small, and none was significant. In addition, noncaregivers reported they had spent more time in vigorous physical activity than caregivers in the prior week,  $F_{(1,61)} = 4.07$ ,  $P = 0.05$ , with caregivers reporting a mean of  $1.44 \pm 2.24$  hr, compared to  $3.58 \pm 5.56$  for noncaregivers; as was true for sleep, amount of exercise was not significantly correlated with immunological data.

The majority of older adults take some medication; in our sample, five caregivers and seven controls took estrogen supplements, five caregivers and six controls took beta blockers or calcium channel blockers, five in each group used prescription diuretics, five caregivers and four controls took thyroid supplements, three in each group used a noninsulin diabetic medication, three in each group used an antiinflammatory medication, two in each group used anxiolytics, and two in each group received antidepressants. These data do not suggest any systematic group differences in medication use.

Similarly, comparisons for each of the health problems from the Older Adults Resources Survey (17) did not produce even marginal differences for any category, and the sum of endorsed problems did not differ between groups,  $F < 1$ ,  $P = 0.37$ . Thus, we found no reliable differences in health-related behaviors

between groups that would have accounted for the immunological differences.

## DISCUSSION

Influenza viruses are responsible for "... more morbidity and mortality than any infectious agent in recorded history" (p. 1700) (7), including considerable "excess mortality" beyond those deaths in which influenza is registered as the cause. For example, a Dutch study (24) showed that for each influenza-registered death there were 2.6 additional influenza-related deaths (47% in people for whom heart disease was the reported cause of death, 23% with lung disease, and 30% with other diseases). Not surprisingly, 95% of these deaths occurred in people who were 60 years or older.

In this study, caregivers and control subjects did not differ with respect to influenza vaccine history, chronic illnesses, medications, income, or age. Nonetheless, we found that caregivers showed clear deficits relative to controls in both their cellular and humoral immune responses to influenza virus vaccine; caregivers were less likely to show a significant increase in antibody titers 4 weeks after vaccination as measured by two independent procedures, and they had lower levels of IL-1 $\beta$  production by monocytes *in vitro*, an important promoter of the antibody response. Caregivers' PBLs also produced lower levels of IL-2 in response to vaccine (antigen) stimulation. The protective capacity of vaccines among the elderly is dependent on their ability to induce both humoral and cell-mediated immune responses (8, 12, 15, 25); both were poorer in the stressed caregivers.

The immunological data from this study are very consistent with the results of studies on stress and influenza infections that have used inbred mice. Restraint stress modulated the immune response to infection by influenza virus in mice, including alterations in the kinetics of the antibody response and the inflammatory response, and the production of virus-induced levels of IL-2 *in vitro* (26, 27). The similarity of data from human and rodent studies provides strong evidence that stress can alter the humoral and cellular immune responses to influenza virus.

Other studies have shown stress-related modulation of vaccine responses in young and healthy subjects. "Academic" stress was related to alterations in both the cellular and humoral immune response to a recombinant hepatitis B virus vaccine in medical students (28), a finding replicated in another study (29). In addition, Cohen *et al.* (30) showed that stress altered susceptibility to several different respiratory viruses in a controlled laboratory study. However, stress-related immunological alterations are likely to have their most potent health consequences in older adults and other at-risk populations who already have impairments in immune function: older adults show greater immunological impairment related to stress or depression than younger individuals (31, 32). Age-related immunological declines are related to the increased vulnerability to infectious illness among the elderly (8, 10, 25).

Stress-related differences in IL-1 $\beta$  may have implications for health beyond infectious disease. Proinflammatory cytokines such as IL-1 play a role in wound healing by preparing injured tissue for repair and enhancing phagocytic cell recruitment (33). We found differences in IL-1 $\beta$  consistent with those reported here in a separate study using an additional 13 caregivers and 13 matched controls (34); in addition, those 13 caregivers took an average of 9 days longer to heal a 3.5-mm punch biopsy wound than controls—, i.e., 24% longer to repair a small, standardized wound.

The differences found in influenza vaccine responses between caregivers and controls provide a well-controlled demonstration of the ways in which stress could alter both the cellular and humoral immune responses to novel pathogens in

older adults. Thus, the data from this study also raise the possibility that chronic stress could have additional ramifications for infectious illness beyond vulnerability to influenza.

We appreciate the technical contributions of Susan Robinson-Whelen, Byron Laskowski, Leigh Ann Kutz, Julianne Dorne, Joseph Rinehart, Wendy Lasekan, Frederick R. Schmitt, Rebecca Brockman-Schneider, and Kit Mui Chiu. Both the vaccine and the purified HA proteins were graciously provided by Connaught Laboratories. This research was supported by Grants R37 MH42096, RO1 MH50538, M01 RR0034, RO1 AG09632, and KO8 AG00548 from the National Institutes of Health and by Ohio State University Comprehensive Cancer Center Core Grant CA16058.

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