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 1β; 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 symptomology 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 γ 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 ± SD of 8.39 ± 8.49 hr per day in caregiving-related activities and reported they had been providing care for an average of 7.25 ± 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 ± SD age was 73.12 ± 8.64 years, compared to 73.30 ± 7.94 years for controls, with a range of 53–89 years. The modal subject reported an annual family income between $20,000 and $30,000.

Abbreviations: HA, hemagglutinin; HAI, hemagglutinin inhibition; IL, interleukin; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte. *To whom reprint requests should be addressed at: 2175 Graves, 333 West Tenth Avenue, Columbus, OH 43210.
were 3044 in parable body Resources system, diabetes, to ized T-cell at had the saccharide for frozen titers 2 Vaccine, Serum (23, 22), cover (18), determined and of other variables. Plasma albumin levels were and (17) show 4-fold variation. 6 months. This selection of vaccine Inventory (but important measured days available and 10% or 95% C02/95% and cytokines. Cytokine analyses. We both ELISAs 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) as substrate, with absorbance 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-1β and IL-6) of monocytes from caregivers and controls. Peripheral blood leukocytes (PBLs) were isolated by density gradient centrifugation on Ficoll-plasmacyte gradients from 40 ml of heparinized venous blood. The isolated PBLs (2 × 10^6 cells per ml) were incubated in 1 ml of RPMI 1640 medium supplemented with 10% male human serum (Sigma) and with 5% Fluzone or Fluzone vaccine (5 μg/ml) containing 11.25, 45.0, or 180 ng of HA protein per ml for 48 hr at 37°C in an atmosphere of 5% CO2/95% air. The Fluzone vaccine was first dialyzed for use as antigen. The cultures were then irradiated with 2000 rads and incubated at 37°C for another 24 hr at which time the supernatant was collected and frozen at −70°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-1β and IL-6 by double-sandwich ELISA. The IL-1β ELISA used a commercially available monoclonal antibody (Genzyme) for coating the plates. Polyclonal rabbit anti-IL-1β 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-1β 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 Ca^2+/-Mg^2+-free phosphate-buffered saline. Approximately 0.5–1.0 × 10^6 cells were aliquoted to 12×75-mm snap-cap tube(s) for reaction with the appropriate monoclonal antibodies: CD3 (total T lymphocytes), T4/T8 (helper/suppressor cells), MO2/KC13 (macrophage/monocytes). Percentages of lymphocyte populations were obtained by gating on lymphocytes; the percentage of macrophage/monocytes was obtained by gating on leucocytes. 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 (60%), 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 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-1β 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+, CD4+, and CD8+ 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+ ($P = 0.96$), CD4+ ($P = 0.56$), or CD8+ 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,
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-1β 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 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-1β 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-1β 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 between groups that would have accounted for the immunological differences.
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.

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