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Influenza in senescent mice: impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection

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SUMMARY

Influenza and pneumonia are leading causes of death in the elderly. Cytotoxic T-lymphocyte activity is responsible for viral clearance after infection and declines with age. We hypothesized that following intranasal infection with influenza virus, aged mice would have decreased anti-influenza cytotoxic T-lymphocyte activity that would correlate with prolonged pulmonary viral shedding. To test this, young (1·5–4·0 month) and aged (22–25 month) BALB/c mice were infected intranasally with influenza A/Port Chalmers/1/73(H3N2). Mice were killed at 3–19 days following infection. Their splenic cytotoxic T-lymphocyte activity was measured by a secondary *in vitro* chromium release assay. Pulmonary viral titres were quantified by growth of titrated lung specimens in fertilized hens' eggs. Serum antibody titres were measured by an ELISA. Young mice responded in a relatively homogeneous fashion. They developed maximal cytotoxic T-lymphocyte activity of $60{\cdot}9 \pm 2{\cdot}0\%$ by Days 11–13, and all except one cleared virus from the lung by Day 7. In contrast, old mice were heterogeneous. Their cytotoxic T-lymphocyte activity peaked at $46{\cdot}9 \pm 5{\cdot}0\%$ and was delayed by 5–7 days. Forty-five per cent were still shedding virus at Days 7 and 8, and shedding persisted for at least 13 days in some mice. There was a strong correlation in both young and aged mice between the presence of virus in the lungs and decreased splenic cytotoxic T-lymphocyte activity ($\chi^2 = 30{\cdot}2$, $P \ll 0{\cdot}001$). No significant difference was found between young and aged animals in serum IgG1 anti-H3 antibody titres. We conclude that following influenza infection in aged mice, impaired cytotoxic T-lymphocyte activity leads to prolonged duration of infection. These observations may lead to a better understanding of the excess morbidity and mortality in elderly persons that occur with influenza.

INTRODUCTION

This study was prompted by three observations. First, influenza has a significant impact on the health of the elderly. Elderly persons have more severe illness and higher attack rates from influenza, which is especially notable in closed populations such as nursing homes. Indeed, in the U.S.A. influenza plus pneumonia is the fourth leading cause of death of persons over age 65.[1] Second, in the experimental setting cytotoxic T-lymphocyte (CTL) activity is clearly responsible for recovery from influenza infections: CTL are both necessary and sufficient for a recovery from influenza pneumonia. This was demonstrated in studies using nude (athymic) mice. Influenza-infected nude mice shed virus from their lungs indefinitely.[2] However, the adoptive transfer of anti-influenza CTL to influenza-infected nude mice will clear the virus from their lungs.[3-4] In contrast, administration of anti-influenza antibody to nude mice will lead to a temporary cessation of viral shedding lasting only as long as antibody is present.[2] Third, CTL activity is decreased in senescent mice following an influenza inoculation.[6-9] Aged mice have decreased CTL activity against viral infected targets, reduced proliferative responses, lower interleukin-2 (IL-2) production, defective antigen presentation, and a decreased number of splenic precursor cells.[6-9] In contrast, B-cell responses to influenza remain relatively intact in aged mice.[10] These important experiments demonstrate the impaired CTL response of aged mice to influenza, but are of limited value as a model of influenza infections in aged humans because parenteral inoculations were used to prime the mice. First, this type of inoculation is non-physiological—influenza is a respiratory tract virus and viremia is a very rare event following influenza infections. Second, the biological significance of the decreased CTL activity was not evaluated.

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Abbreviations: CTL, cytotoxic T lymphocyte; EID_{50}, 50% egg infectious dose; IL-2, interleukin-2.

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Based on the above considerations, we tested the hypothesis that following respiratory infection with influenza virus, aged mice would have impaired development of anti-influenza CTL activity and that this impairment would correlate with prolonged duration of infection. This paper reports the result of these experiments.

MATERIALS AND METHODS

Mice

Young (1·5–4·0 month) and aged (22–25 month) BALB/c NNIA female mice were obtained from Charles River Laboratories (Wilmington, MA). They were housed six/cage, maintained under pathogen-free barrier conditions until used, and fed and watered *ad libitum*. In our laboratory some mice began dying at approximately 23 months and most lived a maximum of 27 months. Animals considered to be moribund were not used, though about 5% died from anaesthesia. No animals had obvious tumours.

Virus

Influenza A/Port Chalmers/1/73 (H3N2) was grown in embryonated chicken eggs as described elsewhere.[11] Its final concentration was approximately $1{\cdot}7 \times 10^7$ 50% egg infectious doses (EID_{50})/ml. In some experiments, influenza B/Georgia (gift of Dr H. Maassab) at 10^5 EID_{50} was used.

Protocol

On Day 0, young and aged mice were anaesthetized with an i.p. dose of 0·02 mg of pentobarbital and infected by the intranasal instillation of 20 μl of H3N2 virus. This effectively infects the total respiratory tract (nares, trachea, and lungs).[11] In the first experiment, mice were killed by cervical dislocation on Days 3, 7, 11 and 13 following infection. In the second experiment, mice were killed on Days 4 (CTL only), 8, 12 and 19.

Following killing, spleens and lungs were harvested aseptically and used for measurement of CTL activity and viral titre, respectively, as described below. Serum was frozen at $-70°$ until assayed for antibody.

CTL assay

The assay was adapted from Bennink *et al.*[12] Spleens were placed in 10 ml of Iscove's modified Dulbecco's medium (Sigma, St Louis, MO) with 10% heat-inactivated foetal calf serum (FCS) supplemented with gentamicin sulphate (50 mg/l), amphotericin B (2·5 mg/l) and 5×10^{-5} M -mercaptoethanol, and single-cell suspensions prepared by grinding with a tissue homogenizer (Fisher Scientific, Pittsburgh, PA). From 2·3 to $5{\cdot}00 \times 10^8$ splenocytes were recovered from young mice and 4·0 to $16{\cdot}0 \times 10^8$ splenocytes from aged mice. Only half the cells were used from aged mice with very large spleens. One-third of the splenocyte suspension was incubated in 100 μl of Eagle's MEM with 20 mM HEPES and 1% FCS (pH 6·75) for 1–1/2 hours with $10^{7{\cdot}5}$ EID_{50} of H3N2 and washed twice. The H3N2-infected splenocytes were mixed in a sterile 80 cm^2 tissue culture flask (Nunc, Roskilde, Denmark) with the remaining two-thirds of the spleen cells in 35 ml of Iscove's medium with 10% FCS and placed in a 95% air/5% CO_2 incubator at 37°. This secondary *in vitro* assay was done in preliminary experiments for 6–10 days and in subsequent experiments for 7 days. At the end of the incubation period, the cells were washed, counted, and resuspended at 3×10^6/ml in Iscove's medium with 10% FCS. Total cell counts ranged from 5·5 to 21×10^6 (young) and 2·7 to 32×10^6 (old).

The target cells were P815, a DBA/2 mastocytoma cell that is histocompatible (H-2^d) with BALB/c mice. The cells were maintained in RPMI-1640 (Gibco, Grand Island, NY) with 10% FCS, gentamicin sulphate (50 mg/l), and amphotericin B (2·5 mg/l) and routinely subcultured three times weekly. On the day prior to the assay, 3×10^6 P815 cells were washed and incubated in 100 μl of MEM with $10^{7{\cdot}5}$ EID_{50} of H3N2 at 37° for 75 min. The cells were washed, resuspended in 5 ml of RPMI-1640 with 10% FCS, and incubated overnight at 37° in 95% air/5% CO_2 incubator. For radiolabelling of target cells, the H3N2-infected P815 cells were resuspended in 100 μl of 5% Tris buffer (Fisher) and incubated with occasional mixing with 200 μCi of Na_2 $^{51}CrO_4$ (ICN Pharmaceuticals, Irvine, CA) at 37° for 1 hr. The H3N2-infected, ^{51}Cr-labelled P815 were then washed three times, resuspended in RPMI-1640 with 10% FCS at 1×10^5 cells/ml, and 100 μl of the cells were added to a 96-well round-bottomed microtitre plate containing triplicate 100-μl samples of serially diluted effector cells. The microtitre plate was centrifuged at 400 *g* for 3 min and then incubated at 37° in a humidified atmosphere containing 95% air/5% CO_2. After a 6-hr incubation, the plate was centrifuged at $\times 1500$ *g* for 1 min and 100 μl of supernatant were removed from each well and placed in individual 12×75-mm plastic tubes. The level of released radioactivity was determined in a gamma counter (Beckman 5500, Palo Alto, CA) and specific lysis calculated from the ^{51}Cr release in counts per minute (c.p.m.) using the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental c.p.m.} - \text{spontaneous c.p.m.}}{\text{maximal release c.p.m.} - \text{spontaneous c.p.m.}} \times 100.$$

Spontaneous and maximal release c.p.m. were determined by incubating 1×10^4 H3N2-infected, ^{51}Cr-labelled, P815 cells with either 100 μl of media or 100 μl of 5% Triton X-100 (Fisher). Spontaneous release of ^{51}Cr in the absence of effector cells was less than 15%.

Virus titration

The lungs were triturated in 2 ml of phosphate-buffered saline (PBS), separated into two equal aliquots, and frozen at $-70°$ until assayed. Virus was detected by injecting triplicate 0·1-ml samples of the triturated lungs into the allantoic cavities of 10-day embryonated chicken eggs that had previously received 0·1 ml of an antibiotic solution containing 250,000 U/ml of penicillin and 250 mg/ml of streptomycin. The eggs were incubated for 3 days at 35°, and the allantoic fluids were harvested and tested for haemagglutination as previously described.[11] If the sample was positive, serial 10-fold dilutions of the other aliquot were injected into eggs in triplicate, and the EID_{50} calculated by the method of Reed & Muench.[13] Log_{10} of undetectable amount of virus was defined as -1.

ELISA

Serum anti-influenza IgG titres were determined by an ELISA. Briefly, serial dilutions of sera were added to 96-well flat-bottomed microtitre plates (Flow Laboratories, Inc., McLean, VA) coated with inactivated H3N2. Goat anti-mouse IgG1 was added, followed by rabbit anti-goat IgG coupled to alkaline

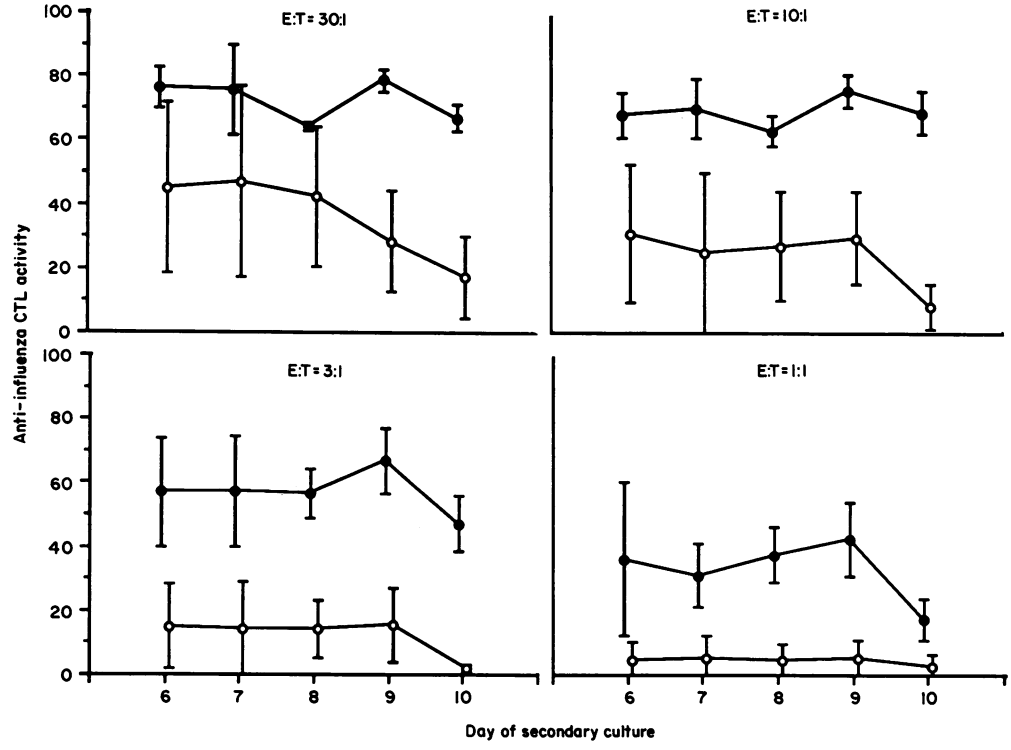

Figure 1. Development of secondary anti-influenza CTL activity of young (●) and aged (○) mice at E:T ratios of 30:1, 10:1, 3:1 and 1:1. Splenocytes were co-cultured with H3N2-infected autologous splenocytes. On the indicated days, a portion of the cells were removed and assayed for anti-influenza CTL activity against H3N2-infected, ^{51}Cr-labelled P815 cells. Points are mean ± SD of percentage specific ^{51}Cr release of five mice per group.

phosphatase and then reacted with p-nitrophenyl phosphate (Sigma). Readings were compared to a standard curve using pooled high-titre sera from animals convalescent from influenza. The pooled high-titre sera were defined as 100 and experimental values were calculated by comparing the dilution of sample to dilution of standard that gave the same OD at 405 nm using a Titertek multiscan (Flow Laboratories) and expressed as a percentage. For example, a titre of 10 is 1/10 the level of the high-titre sera.

RESULTS

Development of H3N2-specific secondary CTL activity

Primary splenic anti-influenza CTL responses after an intranasal infection were very low for both young and aged mice and peaked at < 10% specific lysis at E:T = 100:1 on Days 11–13 (data not shown). Consequently, it was necessary to perform a secondary *in vitro* stimulation of the spleen cells to demonstrate CTL activity. This raised the question of whether the dynamics of secondary stimulation of CTL were different in young and aged mice. Therefore, young and aged mice were studied to determine when maximal secondary CTL activity occurred. Secondary CTL activity was high and fairly constant on Days 6–9 for young mice (Fig. 1). The responses of old mice were significantly lower ($P < 0.05$ at all E:T ratios on all days) than those of young mice, were maximal on Days 6–8, and waned by Day 10. As discussed below, the aged mice also showed greater variability in their response. In the subsequent experiments, 7-day secondary *in vitro* cultures were used.

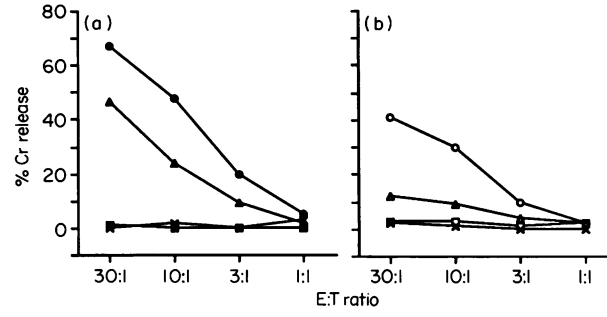

Figure 2. Specificity of CTL from young (a) (closed symbols) and aged (b) (open symbols) mice. Splenocytes from H3N2-infected mice were co-cultured with H3N2-infected autologous splenocytes for 7 days and assayed versus H3N2-sensitized (●, ○), B/Gerogia-sensitized (■, □) or uninfected (X, X) ^{51}Cr-labelled P815 cells. As controls, splenocytes from B/Georgia-infected mice were co-cultured with B-Georgia and assayed versus B/Georgia-sensitized, ^{51}Cr-labelled P815 cells (▲, △). Data are the summary of three experiments of three to five mice/group. Standard errors were < 7% and are left off for clarity.

As demonstrated in Fig. 2, these cells were specific anti-influenza A CTL. As in prior reports on young mice,[14,15] splenocytes from both young and old mice previously infected with H3N2 virus were able to lyse H3N2-sensitized target cells, but not uninfected targets nor influenza B-sensitized targets.

CTL activity following H3N2 infection

Figure 3 demonstrates that young mice have a relatively rapid development of splenic anti-influenza CTL activity following an

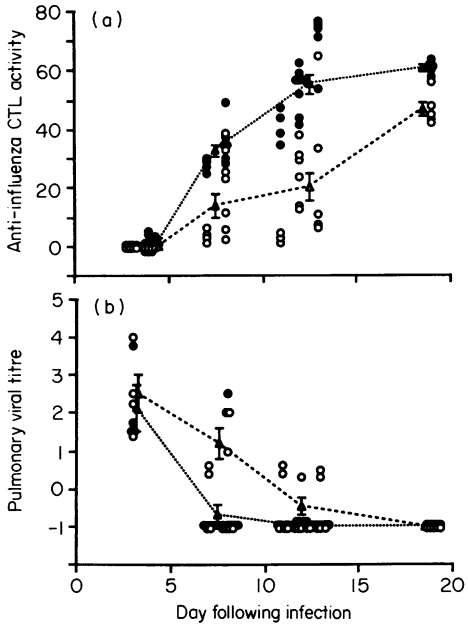

Figure 3. Specific CTL activity (a) and pulmonary viral shedding (b) following an influenza infection in young (●) and aged (○) animals. On the indicated days following infection, splenic anti-influenza activity was measured in a 7-day secondary CTL assay and pulmonary viral titres measured by inoculation of ground lung samples into fertilized hen's eggs. CTL activity is shown as percentage specific ^{51}Cr release at E:T = 10:1. Pulmonary viral titre is $\log_{10}$ of EID_{50}. For simplicity, means ± SE are shown for young (▲) and aged (△) mice for combined Days 3–4, 7–8, 11–13 and 19.

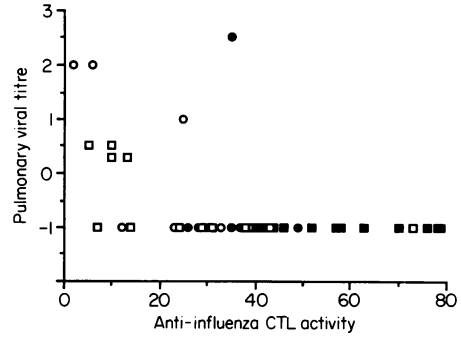

Figure 4. Correlation of secondary anti-influenza CTL activity at E:T = 10:1 and pulmonary viral titres ($\log_{10}$ of EID_{50}) in young (closed symbols) and aged (open symbols) mice on Days 7–8 (●, ○) and 11–13 (■, □). 6/9 mice with < 20% CTL activity shed virus versus 2/29 with > 20% CTL activity. $\chi^2 = 7{\cdot}92$, $P < 0{\cdot}005$.

influenza infection. CTL activity was significant by Days 7–8 and plateaued by Days 11–13 post-infection. Old mice also developed significant CTL activity by Days 7–8, but it was considerably lower than in young mice. Old mice on the average took 5–7 days longer to develop the same level of CTL activity as did young mice. The average maximal CTL activity in aged mice was significantly lower than that in young mice ($P < 0{\cdot}01$ Student's t-test). Using a two-way analysis of variance (age and time), the difference in CTL activity between young and aged mice was highly significant [$F(1,85) = 107{\cdot}7$, $P < 10^{-6}$].

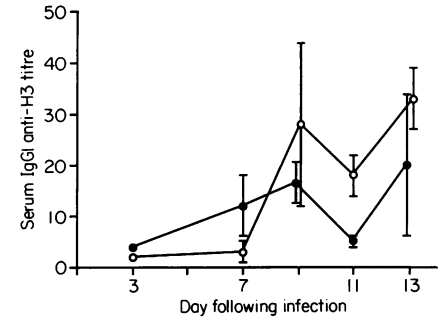

Figure 5. Serum IgG1 anti-H3 titres in young (●) and aged (○) mice following an influenza infection. Values were calculated by ratio of dilution of sample to dilution of reference that gave the same OD reading. Points are mean ± SE.

Also apparent in Fig. 3 is that CTL responses in the aged mice were more heterogenous than in young mice. In fact, the CTL activity in a few old mice was indistinguishable from that of young mice.

Pulmonary viral titres

To measure the severity of infection, we determined the viral content of triturated lungs as a function of time. As shown in Fig. 3, there was efficient clearance of the virus by young mice; all except one cleared virus by Day 7. Aged mice took far longer and showed greater variability in clearing influenza virus from their lungs; some continued to harbour virus in their lungs for at least 13 days. This prolonged viral burden was strongly correlated with diminished splenic anti-influenza CTL activity: 11/18 old mice with < 20% anti-H3N2 CTL were still infected compared with 1/12 with > 20% CTL activity ($\chi^2 = 4{\cdot}02$, $P < 0{\cdot}05$). For both young and old mice, 14/21 mice with < 20% anti-H3N2 CTL activity harboured virus compared with 2/45 with > 20% CTL activity ($\chi^2 = 30{\cdot}2$, $P \ll 0{\cdot}001$). This is illustrated in Fig. 4 for young and old mice from Days 7 and 8 and 11–13. These days were chosen because all animals from both young and aged groups shed virus on Day 3, and none shed on Day 19. On these days 6/9 mice with < 20% anti-CTL activity harboured virus compared with 2/29 with > 20% CTL activity ($\chi^2 = 7{\cdot}92$, $P < 0{\cdot}005$).

Serum antibody

Anti-influenza antibody can speed the recovery from influenza in normal mice.[16] Serum IgG1 anti-H3 antibody titres were therefore measured and are shown in Fig. 5. No significant difference was found between young and aged animals.

DISCUSSION

The elderly suffer excessively from infectious diseases and because of their well-documented decline in immune function, others have speculated that these two phenomena are causally related.[17] However, direct experimental evidence for this relationship is sparse. This may well be because many of the infections predominant in the elderly are of bacterial origin. Though the increased prevalence of bacterial infections in the elderly may be due to the modest age-related decline in

polymorphonuclear phagocytosis,[18] the anatomic and physiologic changes with age—such as diminished pulmonary elasticity, prostatic hypertrophy, and poor skin healing—may be even more important. Impaired immune surveillance may only be an etiological factor in those infections controlled by cellular immune functions, such as tuberculosis,[19] listeriosis,[20] toxoplasmosis,[21] and viral infections.[22]

Of particular interest are previous studies of influenza in senescent mice, in which mice were infected parenterally. These studies showed decreased anti-influenza CTL activity, a 2-day delay in development of CTL activity, defective antigen presentation, lower levels of IL-2 production, and a decrease in splenic precursor cells.[6-9] In the experiments reported in this paper, aged mice infected via the respiratory tract with influenza virus were found to take 5–7 days longer to develop similar levels of splenic anti-influenza CTL activity, had lower levels of maximal CTL activity, and, most importantly, established a strong correlation between decreased CTL activity and prolonged viral infection. Since P815 cells express only class I and not class II molecules,[23] our experiments evaluated the role of putative class I-restricted influenza-specific CTL only. This class of CTL is thought to be most important in the biological clearance of virally infected cells.[24] Recently, however, class II-restricted anti-influenza CTL have been identified, cloned, and shown to reduce pulmonary viral titres in adoptive transfer experiments.[25-27] The exact role of class II-restricted CTL in recovery from viral infections and whether there is a change with ageing remains to be determined.

Our results need to be compared with previously published data on primary and secondary anti-influenza CTL activity of aged mice. Effros & Walford[7] found that after intraperitoneal inoculation of 20–24 month male BALB/c mice, a peak primary CTL response of 40% occurred on Day 5 in young mice and of 30% on Day 7 in aged mice. We found very low (< 10% on Days 11–13) primary CTL activity after respiratory tract inoculation. Effros & Walford (7) also found that, in secondary CTL assays, peak activity occurred on Day 5 for young mice and Day 7 in aged mice. In contrast, we found peak activity on Days 6–10 for young and 6–8 for aged female mice (Fig. 1) although, as with Effros & Walford, the maximal CTL activity was considerably lower in the aged animals. We also noted a larger variability in the responses of aged mice, a commonly observed phenomenon in ageing research.[7,28,29] Because BALB/c mice were used in both experiments, the differences are most likely due to our use of respiratory inoculation to prime the animals, whereas they used parenteral injections, but minor variations in experimental techniques cannot be ruled out (e.g. male versus female mice, or our mice were slightly older).

Our data clearly demonstrate that some aged mice have extremely prolonged pulmonary infection ($\geq$13 days) with influenza virus. Because recovery of influenza virus from the lung indicates that there is histopathological evidence of pneumonia in mice,[30] we postulate that in some elderly persons, infection with influenza virus is also followed by a prolonged viral pneumonia. This would consequently lead to greater likelihood of secondary bacterial pneumonia, cardiopulmonary decompensation, and subsequent increased morbidity and mortality. This is compatible with clinical observations: elderly persons with influenza infections have a mean duration of illness of 13 days versus 3–5 days in younger subjects.[31,32] Further evidence supporting this hypothesis comes from the recent studies of Wyde *et al.*[33] They found that influenza-infected aged mice had a decreased ability to clear bacteria from their lungs. This defect was most closely correlated with the duration of viral infection; no differences were seen in pulmonary macrophage or neutrophil function between young and aged mice.

There are several limitations of our study. First, since they were designed to show a correlation between decreased anti-influenza CTL activity and prolonged illness, they do not address the underlying mechanism of immunosenescence. This subject has recently been reviewed by Thoman & Weigle[29] who point out that there are multiple defects in lymphocytes from aged subjects, especially in such early events as rise in intracellular calcium concentration and activation markers. Further, the molecular mechanisms are not well defined. Despite this caveat, our experiments do make it clear that investigations of decreased CTL function in ageing are not only scientifically interesting, but are clinically relevant.

Second, our studies used splenic CTL activity rather than pulmonary CTL activity. Yap & Ada[34] found that following either an intravenous or intranasal infection of young mice, CTL activity of splenocytes and pulmonary lymphocytes peaked at Day 6 following infection. We are unaware of any literature on the role of pulmonary CTL activity on susceptibility to infection in ageing. This area needs further investigation.

Finally, our studies were done with 'virgin' aged mice, i.e. mice that had not previously been exposed to influenza virus. In practice, most elders have been infected with an influenza A virus when young and then are reinfected with a different strain of influenza A virus when old. We (B. S. Bender and E. Tallman, XIV International Congress of Gerontology, Acapulco, Mexico, June 1989) and others[7] have shown that *in vitro* heterotypic immunity is also lower in aged mice. Clinically, this is an extremely important defence mechanism. Many influenza infections probably start in the upper respiratory tract, and in virgin mice influenza virus can spread from the nose to the lungs in 5 days.[11] The heterotypic immunity of mice previously infected with a different influenza A stereotype, however, is capable of preventing the spread of influenza from the nose to the lung, i.e. it prevents viral pneumonia.[11] We postulate that the decreased heterotypic immunity of aged mice previously infected with H3N2 will allow the spread of a different influenza A serotype (H1N1) from the nose to the lungs. Experiments are currently underway to test this hypothesis.

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