Kinetic Evaluation of the Pool Sizes and Proliferative Response of Neutrophils in Bacterially Challenged Aging Mice

By Gerald Rothstein, Robert D. Christensen, and Buddy Ray Nielsen

Clinical observations during infection suggest that in aged patients, the kinetic or proliferative responses of neutrophils to infection may be deranged. To test this hypothesis, the neutrophil responses of 6-month-old and 30-month-old mice were compared. After intrapulmonary injection of Escherichia coli, young mice exhibited neutrophilia and diminution of the neutrophil storage pool (NSP) by a mean of 6.4 x 10^6 neutrophils/two femurs. This was accompanied by an increase in the pool of CFU-GM from a control value of 1.1 x 10^5 cells/two femurs (range 0.7 to 1.4) to 1.5 x 10^6 (1.1 to 1.9) (P < .05) and the thymidine suicide (relative proliferative rate) of CFU-GM rose from 27% (19 to 42) to 51% (31 to 61) (P < .05). Furthermore, the CFU-GM of infected young mice displayed enhanced differentiation to the neutrophil series. In contrast, old mice exhibited a greater mean diminution of the NSP: 12.8 x 10^6 neutrophils. Also, old mice experienced a reduction in CFU-GM from 2.3 x 10^6 (1.0 to 3.9) (controls) to 1.3 x 10^6 (1.2 to 1.5)/two femurs (P < .05), a reduction in the proliferation of CFU-GM and reduced differentiation of CFU-GM to neutrophils. These experiments establish that the neutrophil response of infected old mice is disordered, with exaggerated depletion of the NSP and lack of stimulus-driven granulocytopenia as reflected by a paradoxical reduction in the number and proliferative rate of precursors. This defect may be compounded by decreased differentiation of precursors to neutrophils.

MATERIALS AND METHODS

Animals. Six-month-old or 30-month-old male C57Bl/6 mice were obtained from Charles River Laboratories (Stone Ridge, NY) under the auspices of the National Institute of Aging. Each of the animals was shipped in an individual one-mouse compartment and was housed individually at the University of Utah Vivarium until use within 1 to 6 weeks of arrival. When killed, animals were examined by autopsy for gross evidence of tumors, renal disease, hepatic disease, or inflammatory lesions. Animals exhibiting these abnormalities were excluded from the study.

Examination of the blood and bone marrow. Mice were narcotized by CO2 inhalation, and blood was obtained from the vena cava. The nucleated cells were counted electronically, and differential counts of 100 cells were performed on Wright's stained smears. Absolute concentrations of neutrophils were calculated from the nucleated cell counts and differentials.

Bone marrow was flushed from both femurs according to the method of Chervenick and colleagues. Cells were counted electronically and 500-cell differential counts were performed, using Wright's stained smears and established criteria for identification of cells. Absolute numbers of the various marrow cells were calculated from the nucleated cell counts and differentials. All studies of hemic cells involved killing the animal; consequently, no serial measurements were performed in individual mice.

Cultures of CFU-GM. Cultures of CFU-GM were performed in 35-mm plastic dishes, using semisolid methylcellulose medium in a manner previously described. Cultures contained 5% heat-inactivated serum, a concentration that exerted maximal stimulatory effect for the formation of colonies from both young and old mice. Cultures were incubated for 7 days at 37°C in a 7% CO2 atmosphere. Colonies (>50 cells) were scored with the aid of a stereomicroscope.

Thymidine suicide experiments were carried out in a manner similar to that described by Byron. Marrow cells were incubated at 37°C for 20 minutes in 2 ml of Hank's Balanced Salt Solution (HBSS) containing 0.1 mCi [3H]TdR (New England Nuclear, North...
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Billericia, MA) with a specific activity 80 Ci/mmol. Corresponding control cells were incubated in an equivalent amount of 3HTdR. 3HTdR uptake was inhibited competitively by adding 30 mL cold HBSS with 15% fetal calf serum (FCS) and 100 g/mL 3HTdR. The cells were centrifuged at 200 × g for 12 minutes and were washed twice in the HBSS-cold thymidine serum solution. The thymidine suicide was taken as the proportional decrement in colonies when CFU-GM were pretreated with 3HTdR, as compared with 3HTdR.

The morphology of colonies was examined by aspirating all clearly isolated colonies from culture dishes with a capillary pipette, and then smearing them on coverslips for Wright’s staining. Differential counts were performed on at least 50 cells for each colony; if sufficient numbers of cells were present, 100 cells were counted.

E coli expressing the K1 antigen were kindly supplied by Dr Harry Hill, University of Utah. Organisms were grown overnight in Tryptic Soy Broth (TSB) and washed three times in phosphate-buffered saline (PBS) prior to resuspension at 10⁶ to 10⁸ CFU/μL in PBS for injection with a Hamilton syringe. Concentrations of E coli were estimated by measuring the absorbance of suspensions at 600 nm, and the numbers of viable CFU were verified by quantitative culture in TSB agar.

Data were evaluated statistically with Student’s t test, the Wilcoxon rank-sum test, or the Fisher exact test. Considering the usual sample sizes of four to eight values, these maneuvers are effective in evaluating the validity of apparent differences but could fail to detect more subtle differences that might not achieve statistical significance with relatively small numbers of values.

RESULTS

Weights of the 53 young mice (33.34 ± 0.32 g) (mean ± SEM) and the 50 old mice (33.29 ± 0.42 g) did not differ.

Mortality after inoculation with E Coli. Six-month-old (young) animals were inoculated transthoracically into the right lung with 1 μL/g body weight of a PBS suspension containing various doses of E coli. All five animals that received 10⁶ E coli/g died within 48 hours. Of ten mice that received 10⁵/g, six died within 48 hours, and the remainder survived until they were killed after 96 to 116 hours. No mortality occurred in 53 young mice or 50 old mice that received 10⁵ E coli/g, nor were there any deaths among 17 mice after injection with 1 μL/g of PBS.

Effect of bacterial challenge (10⁴ CFU/g) on circulating and marrow neutrophils. Four noninfected young mice displayed blood neutrophil concentrations of 1.6 × 10⁶/μL (0.7 to 2.1) (mean and range); four old controls exhibited neutrophil concentrations of 2.4 × 10⁵ (2.0 to 2.9) (P = .057). The peak concentration of neutrophils was observed 2 hours after inoculation for both groups; four young mice had a peak of 3.3 × 10⁵ (2.4 to 4.6) neutrophils per μL; four old mice exhibited a peak of 6.2 × 10⁵ (3.3 to 11.3)/μL (P = .057). After 6 hours, the neutrophil concentrations for both groups had returned to their control ranges.

The mean value for the marrow’s NSP (metamyelocytes plus band plus segmented neutrophils) of noninfected old mice was 47% larger than that for young control animals (Fig 1). After bacterial inoculation, the NSP diminished to a mean of 12.8 × 10⁶ cells/two femurs (59% depletion); whereas the young animals experienced a smaller depletion: a mean of 6.4 × 10⁶ cells/two femurs (43% depletion). Twenty-four hours after inoculation, the NSP of young animals had begun significant repletion; by 48 hours, the number of cells in the NSP had returned to the range of control values. In contrast, the NSP of old animals remained significantly smaller than that of noninfected controls, and was not repopulated even after 48 hours (P < .005).

Four hours after inoculation, the pool of morphologically recognizable proliferative precursors (blasts, promyelocytes, and myelocytes) was unchanged in young animals, but after 48 hours had increased to a mean of 145% of that for controls (P = .02) (Fig 2). In contrast, after 4 hours, the proliferative pool of old animals was reduced to a mean of 56% of the control value (P = .05) and remained depressed at 24 hours (Fig 2). Forty-eight hours after inoculation, old animals exhibited repletion of the proliferative pool to control values. As controls, 11 old mice and six young mice received injections of 1 μL PBS/g and did not exhibit neutrophilia or alterations in the marrow cellularity.

Changes in the number and proliferative rate of CFU-GM. In young mice, the femoral pool size of CFU-GM was unchanged at 4 hours but after 48 hours, had increased to a mean of 145% of the control value (Fig 3). In old mice, the mean number of CFU-GM fell to 46% of the control after 4 hours and remained reduced (55% of control) at 48 hours.

The thymidine suicide of CFU-GM for eight young control mice was 27% ± 3%, and for 6 old control mice it was 39% ± 4% (P = .054) (Fig 4). Thereafter, bacterial chal-
Fig 2. Effect of E. coli inoculation on the proliferative pool of granulocytes (blasts, promyelocytes, and myelocytes). Means for groups of four to seven 30-month-old animals (○) designate the means for groups of four to eight 6-month-old animals. Brackets indicate SE. *P < .003 v 6-month-old controls; **P < .05 v 30-month-old controls; and †P < .02 v 6-month-old controls.

Fig 3. Effect of E. coli inoculation on the number of CFU-GM. Mean values for groups of four to seven animals, aged 30 months (○); mean values for groups of four to eight 6-month-old mice (●). Brackets indicate SE. *P < .04 v 6-month-old controls; **P < .05 v 30-month-old controls; and †P < .02 v 6-month-old controls.

Fig 4. Effect of E. coli inoculation on thymidine suicide of CFU-GM. Means for groups of four 30-month-old animals (○); mean values for groups of four 6-month-old animals (●). Brackets indicate SE. *P < .04 v 6-month-old controls; **P < .002 v 6-month-old controls; †P < .08 v 30-month-old controls, ‡P < .002 v 6-month-old at 48 hours.

Challenge induced distinctive and different alterations in the thymidine suicide of CFU-GM from the two groups. After 4 hours, the mean thymidine suicide of inoculated young animals had risen (P < .04), achieving a peak mean value of 51% ± 4% at 48 hours (P < .002 v control). In contrast, in old animals, the thymidine suicide of CFU-GM fell within 4 hours and at 48 hours was still depressed as compared with that of 30-month-old controls (P < .04) or 6-month-old animals at 48 hours (P < .002).

Next, the effect of a larger, ~50% lethal, bacterial inoculum was tested. Twenty-one young mice were given 10^8 E. coli/g; 10 died within 48 hours. Six old mice received the same weight-standardized dose and three died within 48 hours. Forty-eight hours after inoculation, CFU-GM from four young survivors and three old survivors were evaluated (Table 1). Young mice responded to the larger inoculum with an even greater increase in the number of CFU-GM as well as an increase in the thymidine suicide of CFU-GM. In contrast, old mice displayed a reduced population and thymidine suicide of CFU-GM. In aged mice, the high dose of bacteria induced a significant further decrement in the number of CFU-GM as compared with the smaller nonlethal dose of 10^5/g (P < .05) (Table 1).

Morphological studies of the progeny of CFU-GM in vivo. Twenty-eight to 38 colonies were aspirated from 7-day-old cultures and examined morphologically. In this way, the proportion of neutrophils within colonies was determined for groups of three to four old or young animals. The data for control animals and for animals who had received E. coli 48 hours previously are shown in Table 2. With bacterial challenge, young animals significantly decreased the propor-
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In each group are shown in brackets. Ranges are shown in parentheses. Numbers of experiments are constant pools of proliferative and stored mature neutrophils colonies evaluated for the respective categories. Granulocyte kinetics by a standardized infectious challenge, and the neutrophil supply is restored.2 These are sustained.7 During infection, the system is perturbed. Utilization of neutrophils are balanced, so that relatively increasing the blood neutrophil pool and decreasing the size of neutrophilia in 24-month-old and 6- to 8-month-old mice. The size of the pool of immature granulocytes was undoubtedly influenced by movement of immature cells into the NSP. This was accompanied by a reduction in the number of CFU-GM however, suggesting that reduced

Table 1. Effect of E coli Inoculation on Number and Thymidine Suicide Rate of CFU-GM

<table>
<thead>
<tr>
<th>Mice</th>
<th>CFU-GM x 10^2/2</th>
<th>Thymidine Suicide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 months old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (8)</td>
<td>1.1 (0.7-1.4)</td>
<td>27 (19-42)</td>
</tr>
<tr>
<td>10^3 E coli/g</td>
<td>1.5 (1.1-1.9)*</td>
<td>51 (31-61)*</td>
</tr>
<tr>
<td>10^4 E coli/g</td>
<td>1.9 (1.5-2.1)†</td>
<td>49 (43-53)*</td>
</tr>
<tr>
<td>30 months old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (6)</td>
<td>2.3 (1.0-3.9)</td>
<td>39 (28-55)</td>
</tr>
<tr>
<td>10^3 E coli/g</td>
<td>1.3 (1.2-1.5)*</td>
<td>24 (18-36)*</td>
</tr>
<tr>
<td>10^4 E coli/g</td>
<td>1.0 (0.8-1.1)‡</td>
<td>28 (25-29)*</td>
</tr>
</tbody>
</table>

Means are given for control mice and those injected with E coli 48 hours earlier. Ranges are shown in parentheses. Numbers of experiments are shown in each group are shown in brackets.

*p < .05 v control.
†P < .05 v value for 10^4/E coli.
‡P = .028 v 10^4 E coli/g 30-month-old mice and 10^4 E coli/g 6-month-old mice.

In noninfected animals and humans, the production and utilization of neutrophils are balanced, so that relatively constant pools of proliferative and stored mature neutrophils are sustained.7 During infection, the system is perturbed. Cells are acutely released from the NSP into the blood, increasing the blood neutrophil pool and decreasing the size of the NSP.12 In compensation, there is an increased proliferative effort, and the neutrophil supply is restored.2 These kinetic and proliferative responses depend on an intact system of regulatory stimulators (or inhibitors) and target cells capable of an appropriate response. The present studies were carried out on the premise that during perturbation of granulocyte kinetics by a standardized infectious challenge, the regulation of neutrophil kinetics and proliferation could be evaluated in groups of young and aging animals.

First, we examined pool sizes and proliferation in the steady state. Femoral marrow was studied quantitatively, and the data were taken to reflect total marrow cellularity, since previous measurements indicate a similar marrow distribution in young and old mice.6,11 The total marrow cellularity of old mice was greater than that of young animals. These data agree with those of Boggs et al.10 Williams and co-workers also noted an increase in marrow cellularity in old mice, but this difference was not apparent when values were adjusted for the animals' body weight.9 In our experiments, the old mice also exhibited an increased NSP, an increased proliferative pool, and an increased pool of CFU-GM. None of these increases were due to differences in body mass, because young and old animals did not differ in weight. In addition, old mice exhibited an increased thymidine suicide of CFU-GM. Because the time for DNA synthesis is fixed in mammalian cells,11 an increase in thymidine suicide is considered a reflection of a shortened cell cycle duration and therefore an increased rate of proliferation. Thus, baseline neutrophil production may be greater in old mice as compared with young adult mice.

After a nonlethal challenge with E coli (10^4/g), young mice exhibited a reduction in the NSP and neutrophilia as neutrophils were released into the blood. Then the population of immature granulocytes and of CFU-GM increased, and the thymidine suicide rate of CFU-GM also increased. The increase in proliferative effort is the consequence of stimulus-driven granulocytopenia, which facilitates repopulation of the NSP.

Old mice exhibited a greater degree of neutrophilia than did young animals. Esposito and Pennington noted similar degrees of neutrophilia in 24-month-old and 6- to 8-month-old mice challenged with bacteria although the older mice exhibited slightly but consistently lower values.12 We are hesitant to compare our results with theirs, however, because in the present study older mice and a different organism were used. In our experiments, inoculated old mice lost a greater number of neutrophils from their NSP, and the proportional depletion of the NSP was also greater. These data reflect an intact stimulus and response for neutrophil release, but one resulting in a greater degree of depletion of the NSP in old mice. Even so, neutrophil supply was not sufficiently compromised at the dose of 10^6/g to induce neutropenia. Neutrophil counts were not measured after the high dose of E coli. Kinetically, the exaggerated depletion of the NSP is the net consequence of inflow of neutrophils to the NSP from the mitotic compartment and exit of cells into the blood. We did not directly measure neutrophil release, but our studies suggest that diminished proliferation contributes to the exaggerated depletion and to delayed repletion of the NSP. In support of this, the population of immature granulocytes fell acutely in old animals and then returned to control levels rather than remaining stable and then rising as it did in young mice. The size of the pool of immature granulocytes was selected for the animals' body weight.

Table 2. Morphology of CFU-GM Colonies in Control Mice and Mice Inoculated With E Coli 48 Hours Previously

<table>
<thead>
<tr>
<th>Neutrophils in Colonies (%)</th>
<th>6 Months Old</th>
<th>30 Months Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (36)</td>
<td>Control (36)</td>
</tr>
<tr>
<td></td>
<td>E coli 10^3/g</td>
<td>E coli 10^3/g</td>
</tr>
<tr>
<td>0</td>
<td>25%</td>
<td>18%</td>
</tr>
<tr>
<td>1-33</td>
<td>42%</td>
<td>42%</td>
</tr>
<tr>
<td>34-66</td>
<td>19%</td>
<td>25%</td>
</tr>
<tr>
<td>67-100</td>
<td>14%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Values are for proportion of colonies in each category; numbers of colonies evaluated for the respective categories are shown in parentheses.

*p < .05 v 6-month-old controls or 30-month-old 10^6/g.
†P < .05 v 30-month-old controls. P < .001 v 6-month-old 10^6/g.
‡P < .03 v 6-month-old 10^6/g.
inflow into the immature cell pool was also a factor. The significance of a reduced pool of CFU-GM appears to be compounded by a reduction in the differentiation of CFU-GM to the neutrophil series in vitro. In contrast, in inoculated young mice, differentiation to neutrophils was increased. An even more pronounced fall in the number of CFU-GM was observed in old mice given the high dose of $10^9$ E coli/g, whereas the high dose of E coli yielded a further increase in CFU-GM in young mice. The reduction in the pool of GFU-GM was not accompanied by an increase in the proliferative rate of CFU-GM, which actually fell after both the high and low doses of E coli. Therefore, inoculated old mice did not respond with increased granulocytopoiesis. Although the mechanisms responsible for this are not clear, candidate explanations include insufficient production of a necessary stimulator, production of an inhibitor of proliferation, or hyporesponsiveness of precursors to stimulators.

It has been proposed that during aging, mitotically capable cells lose their ability to proliferate. Hayflick has demonstrated loss of replicative potential in senescent diploid cell lines, and Dell'Orco et al correlated the life span of fibroblasts with their number of cell doublings. In addition, Albright and Makinodan showed that hematopoietic stem cells from old animals produced relatively few spleen colonies in irradiated recipients. Lipschitz et al demonstrated reduced numbers of CFU-GM, BFU-E, and CFU-E in elderly anemic subjects, and reduced iron incorporation by cells from old animals produced relatively few spleen colonies derived from 3- to 5-month-old mice housed in groups. The kinetics of the neutrophil response and its effect on proliferation have not been evaluated in group-housed mice, and the factors responsible for depressing precursors are not at all clear. In the present study, mice were shipped and housed singly. Despite these precautions, we cannot exclude the possibility that factors such as shipping (or other factors unknown to us) may have influenced or primed the hematopoietic system of aging mice, thus contributing to their response to infection.

We applied a standardized stimulus to evaluate neutrophil kinetics in bacterially challenged old mice. The response of old mice differed from that of young animals, and was characterized by (a) an exaggerated depletion of the NSP, (b) a paradoxical diminution of the pool sizes of CFU-GM and their progeny, (c) a paradoxical lack of accelerated proliferation of CFU-GM, and (d) a diminished differentiation of CFU-GM to neutrophils. These changes reflect an absence of the expected stimulus-driven granulocytopoiesis which appropriately increases the neutrophil supply in young mice. As yet, the effect of this abnormality on host resistance is unknown, although in the present short-term experiments, no increase was observed in the mortality of infected aged animals. We propose that the model of bacterially challenged aged mice may be useful in identifying the mechanisms responsible for these abnormalities.

REFERENCES

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