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Inflammation, Vol. 20, No. 6, 1996

NEUTROPHIL MIGRATION, OXIDATIVE METABOLISM, AND ADHESION IN ELDERLY AND YOUNG SUBJECTS

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Abstract—Objective. To evaluate neutrophil functions in the elderly. *Methods.* We investigated the PMN migration in vivo and PMN superoxide production and adhesion in response to a variety of compounds; PMN have been isolated both from blood and from a skin experimental exudate (obtained by Senn's skin window technique) of 25 normal elderly and of 25 normal young control subjects. *Results.* No difference was found in PMN migration in vivo ($62.9 \pm 21.3 \times 10^6$ and $65.5 \pm 9.1 \times 10^6$ PMN/cm^2/24 hours in elderly and young subjects respectively), neither were different the adhesion under basal condition and after some stimuli and the superoxide production in basal condition and in response to STZ and PMA in two groups. In elderly subjects superoxide production, in response to fMLP, markedly resulted lower than in young controls both by circulating PMNs (3.6 ± 2.7 and 9.3 ± 3.3 nMOLES $O_2^-/10^6$ PMN respectively, $p < 0.0001$) and by exudate PMNs (13.6 ± 4.3 and 19.4 ± 6 nMOLES $O_2^-/10^6$ PMNs respectively, $p < 0.005$). *Conclusion.* Many PMN functions in the elderly do not differ from young people, suggesting that the overall defense function of these cells is not affected by aging. The only parameter that we have found to be different between the two groups is the poor superoxide production after fMLP stimulus of PMNs. The stimulus- and function-specificity of this defect in PMNs from elderly subjects indicates the existence of a dysregulation of the signal transduction pathway distal to fMLP receptor and proximal to NADPH oxidase activation.

INTRODUCTION

Neutrophils (PMN) are the first cells that migrate into tissues in response to invading pathogens. The accumulation of these cells at sites of inflammation is

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accompanied by modification of their activity (ability to release granule constituents and toxic oxygen derivatives, adhesion capacity etc.).

Previous investigations on human models have shown that exudate PMNs are metabolically primed, being more responsive to various membrane stimulants, than blood PMNs (1–7). The increased activation state of PMNs is relevant for non specific immunological surveillance against microbial invaders and also because of the potentially harmful role played by these cells in pathologic processes as adult respiratory distress syndrome, postischemic tissue disease, immunocomplexes disease etc. (7–10).

During the last few years some reports have evaluated the PMN's functions in the elderly. The results are discordant. Some studies have shown that the functional response of PMNs is unaffected by aging (11), while others have pointed out an impairment of chemotaxis, phagocytosis and killing (12–14), a reduction of respiratory burst (15–17) and a decrease of adhesion (15).

These studies have been always performed in vitro, utilizing PMNs isolated from blood.

In the present study we have investigated the PMN migration in vivo and PMN superoxide production and adhesion, in response to a variety of compounds; PMNs have been isolated both from blood and from a skin experimental exudate (obtained by Senn's skin window technique) of normal elderly and of normal young control subjects.

MATERIALS AND METHODS

Twenty-five volunteers (12 M, 13 F) over 65 years old (mean age 76.56 ± 7.36 years, range 68–100 years), were selected according to SENIEUR protocol (18). Twenty-five volunteers young sex matched subjects (mean age 27.77 ± 2.59 years, range 22–34 years) were considered as controls.

During the 3 weeks before the evaluation, no subjects were taking NSAIDs, corticosteroids or any other drugs.

All the elderly subjects were in good nutrition state.

Reagents. The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), zymosan and phorbol myristate acetate (PMNA) were purchased from Sigma Chemical Company, St. Louis, Missouri; cytochrome *c* from Boerhringer, Mannheim, Germany; purified serum bovine albumin and human albumin from Behring Institut, Marburg, Germany. Percoll was from Pharmacia, Uppsala. Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. Hank's balanced salt solution (HBSS) and reagents were of the highest purity available. Zymosan, opsonized with a pool of normal human sera (STZ), as described by Metcalf et al. (19), was stored in aliquots at −20°C.

In order to avoid contamination, a possible cause of artifactual activation or priming of the cells, sterile apyrogenic solutions and disposable plasticware were used in all experiments, which were carried out, whenever possible, under laminar flow hood. Reagents were prepared using pyrogen-free water or 0.9% NaCl solutions.

Cell Preparation. PMNs were obtained from blood and from skin window exudates of subjects after having obtained their informed consent. Blood PMNs were prepared from 40 ml of

ethylene diamine tetraacetate-anticoagulated blood by centrifugation over Percoll gradients (19). Cells (>95% neutrophils, >95% trypan-blue negative) were finally suspended in HBSS containing 5 mM glucose and 0.2% human serum albumin (HGA) and kept at room temperature until use. A few minutes before use, 100× concentrated solutions of $CaCl_2$ and $MgSO_4$ were added to the cell suspensions at the final concentration of 0.5 mM and 1 mM respectively.

Exudate neutrophils (>90% neutrophils, >95% trypan-blue negative) were isolated according to the method described by Senn (20), with modifications (3). The volar surface of nondominant forearm was disinjected with ether and an abrasion of 1 cm^2 was obtained with a rotating sterile abrasive cylinder operated by a milling cutter (minidrill, Saint Julien en Genevois, France).

The abrasion did not cause bleeding as only the epidermis was removed and the wet, transuding surface of derma was exposed. A bell-shaped, sterile and disposable plastic skin chamber with circular adhesive base (FAR Italia, Verona, Italy) was put on the skin abrasion and fixed with a fenestrated sticking plaster. The chamber has on its top a 5-mm-wide hole equipped with a plug. One ml of autologous serum was then injected into the chamber and twenty-four hours later the exudate was collected by aspiration. The exudate cells were then centrifuged at 1200 rpm, washed twice with phosphate buffered saline (PBS) and finally suspended in HGA and kept at room temperature until use. Before use, the cell suspensions were supplemented with $CaCl_2$ and $MgSO_4$ as described above for blood cells.

Oxidative Metabolism Assay. Superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (21) in a microplate assay system. Assay were performed in order to compare cells from blood and from exudate of the same subject and to compare cells from young and elderly subjects in the same day and using the same microplate in matched replicate wells. The microplate assay for O_2^- production was performed according to previously published methods (22), with the following modifications. The wells were coated with fetal bovine serum, then were supplemented with 25 μl of 0.6 mM cytochrome *c* in HGA containing 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$ (HGACM) and with 25 μl of the stimulants diluted in HGACM at a concentration exactly 4 times higher than that required in the assay. This was done because each agent is diluted 4 times in the final incubation mixture (see below). The plate was then brought to 37°C, and 50 μl of the PMN suspensions (2×10^5 cells), pre-warmed at 37°C, were added to each well using a multichannel pipette and the plates were incubated at 37°C for the desired time. Assays were done in triplicate for each experimental condition. At the time indicated, the plates were rapidly transferred into a microplate reader (Reader 400 SLT Labs Instruments) and the reduction of cytochrome *c* was measured at 550 nm using 540 nm as reference wavelength, and using 0.037 optical density units as standard for 1 nmole of reduced cytochrome *c* (22).

Adhesion Assay. For adhesion measurements, the plates were transferred to an automatic washer (Easy Washer 2 SLT Labs Instruments) and subjected to two washing cycles with PBS at room temperature. Adherent cells were quantitated by measuring the membrane enzyme acid phosphatase and the percentage of adhesion was calculated on the basis of a standard curve obtained with known numbers of PMNs (22).

Statistics. The non-parametric Wilcoxon test for unpaired data (Mann-Whitney test) was used for statistical analysis.

RESULTS

Figure 1 reports the data concerning the values of in vivo migration. The mean of the migration was $62.9 \pm 21.3 \times 10^6$ PMN/cm^2/24 hours in elderly group and $65.5 \pm 9.1 \times 10^6$ PMN/cm^2/24 hours in controls. The difference

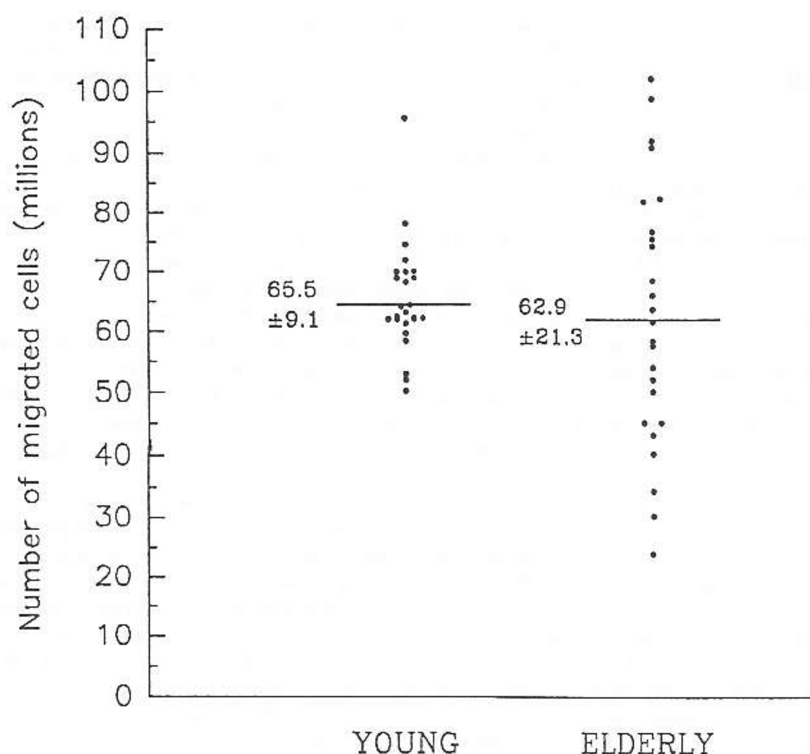

Fig. 1. Data concerning the values of in vivo migration. The mean of the migration was 62.9 ± 21.3 × 10^6 PMN/cm^2/24 hours in elderly group and 65.5 ± 9.1 × 10^6 PMN/cm^2/24 hours in controls.

between the mean migration in the two groups was not statistically significant, but elderly subjects showed higher variation and a more scattered distribution of individual values.

Table 1 reports the data about superoxide production by circulating and migrated PMNs, under basal condition and after challenge with various stimuli (fMLP, STZ and PMA). Exudate cells of both elderly and young groups showed an increased superoxide production, in response to fMLP, as compared with blood cells. This priming effect was not observed using STZ or PMA as stimulants.

Circulating PMNs from elderly subjects showed superoxide production after treatment with fMLP markedly lower than young subjects. Also the exudate cells from elderly subjects had lower activity with respect to exudate cells of young subjects, but the difference between the two subject's groups was not so marked as for blood cells. In fact the net increase of oxidative metabolism caused by migration into the inflammatory exudate (i.e. the priming effect) was similar

Table 1. Superoxide Production in Young (Y) and Elderly (E) Subjects by Circulating (B) and Migrating (SW) PMNs[a]

Stimulants		Y	E	p
None	B	0.7 ± 0.8	0.3 ± 0.4	NS
	SW	1.1 ± 1.2	0.8 ± 0.9	NS
fMLP	B	9.3 ± 3.3	3.6 ± 2.7	<0.0001
10^{-7} M	SW	19.4 ± 6	13.6 ± 4.3	<0.005
STZ 0.1	B	8.2 ± 3.2	8.4 ± 6.1	NS
mg/ml	SW	8.6 ± 2.8	8.9 ± 5.6	NS
PMA	B	21.5 ± 6.2	21.2 ± 6.7	NS
10 ng/ml	SW	21.7 ± 6.1	24.1 ± 7.6	NS

[a]The values are expressed in nmol/10^6 neutrophils.

Table 2. Adhesion of Circulating (B) and Migrating (SW) Neutrophils in Young (Y) and Elderly (E) Subjects[a]

Stimulants		Y	E	p
None	B	12.4 ± 12.1	11.2 ± 8.1	NS
	SW	24.1 ± 19.1	19.5 ± 10.7	NS
fMLP	B	26.3 ± 11.9	23.9 ± 15.5	NS
10^{-7} M	SW	32 ± 17.7	31.4 ± 19.1	NS
STZ 0.1	B	26.6 ± 13	29.2 ± 10.3	NS
mg/ml	SW	35.7 ± 14.6	36.9 ± 16.9	NS
PMA	B	51.2 ± 12.2	49.1 ± 13.6	NS
10 ng/ml	SW	52.9 ± 12.2	49.6 ± 17.8	NS

[a]The values are expressed in percentage of adherent cells.

in the two groups (from 9.3 to 19.4 and from 3.6 to 13.6 in young and elderly subjects respectively). The other values of superoxide production in response to STZ and PMA were not different between the two groups.

Table 2 shows the results concerning adhesion of circulating and migrating neutrophils under basal condition and after some stimuli. Exudation caused a slight increase of adhesion of both unstimulated and stimulated cells. There was no difference between elderly and young subjects.

DISCUSSION

Previous studies on PMN function in elderly subjects have shown decrease of chemotaxis, phagocytosis and killing after some stimulants (12–14). Recently

a reduced superoxide production after fMLP stimulus has been described; it has been hypothesized that this defect may be due to a reduced receptor expression on cellular surface (23–25) or to a deficiency of calcium homeostasis (17) or to an impairment of neutrophil membrane fluidity (26). A further aspect of neutrophil functional deficiency in elderly subjects is the reduced superoxide production by cells primed in vitro (27). A reduced ability of adhesion to plastic surface treated with proteins has been demonstrated (15). All the data have been obtained by experiment in vitro and, to our knowledge, no studies have been carried out in vivo. Moreover, in no other study, the oxidative metabolism and the adhesion have been evaluated simultaneously and in the same experimental conditions.

The skin window technique allows us to evaluate the PMN migration in vivo and to obtain cells migrated from an inflammatory focus: they are characterized by the changes physiologically provoked by phlogosis processes. Moreover our method permits to compare in a simultaneous assay various functional parameters of circulating and migrating PMNs from the same subject.

In vivo migration of neutrophils was quantitatively in the normal range in our elderly subjects group, indicating that neither endogenous generation of chemotactic compounds, nor endothelial and tissue components involved in cell migration, nor neutrophil movement machineries are affected by aging process.

In accordance with previous investigations from our and other laboratories (1–7) our results show that exudate PMNs are metabolically primed, being more responsive to fMLP stimulus than blood neutrophils and show increased adherence in the absence of stimulants. This work shows that the priming event occurs also in elderly subjects. In fact the net difference of oxidative responses to fMLP between exudate and blood cells is similar in the two examined groups, and this is in agreement with the normal chemotactic migration observed in vivo in elderly subjects.

Our results do not perfectly agree with the literature data: in fact, in our study, PMN migration and adhesion resulted similar in young and elderly subjects. PMN migration is a very complex phenomenon, based on both the integrity of adhesive PMN function of cytoskeleton dynamics and the action of chemical substances; a skin window method permits to evaluate this phenomenon in a global way, unlike in vitro techniques that study only the chemotaxis. It is conceivable that in vitro evaluation of chemotaxis, that is carried out under specific conditions, might unmask specific defects that are not relevant for in vivo migration.

Instead, our study falls into line with previous data as regards the lower superoxide production in elderly than in young by circulating PMNs after fMLP stimulus; we did not observe any difference by utilizing other stimuli. Therefore the PMN defect of elderly subjects seems to be both stimulus specific (fMLP

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versus STZ and PMA) and function-specific (superoxide production versus migration and adhesion).

A defect of NADPH oxidase seems to be excluded, because the PMN response after PMA, that directly operates on protein kinase C, and to STZ, that operates through distinct receptors and transduction pathways, is similar in the elderly and young groups.

Some authors have demonstrated a diminished expression of fMLP receptors on the PMN membrane in elderly subjects (23–25). We did not measure fMLP receptors in our study, but our data, showing that fMLP-stimulated adhesion and in vitro migration are normal, suggest that if a deficiency of chemotactic receptors is present, this is relevant only for some functions (oxidative metabolism) and not for others (adhesion and migration). However, this possibility seems to be excluded because we have previously showed that oxidative metabolism activation and adhesion have similar dependence on fMLP doses (6, 22).

According to our data, the stimulus- and function-specificity of blood PMN's defect in elderly subjects appears to be due to inhibition of some mechanism located in the signal transduction pathway between fMLP receptor and NADPH oxidase.

The precise mechanism responsible for these age-related differences remains to be identified, but an hypothesis can be advanced on the basis of our previous findings using inhibitors of leukocyte activation. In fact, we have demonstrated (22) that adenosine, a physiological nucleotide that is known to cause increase of intracellular levels of cAMP (28, 29), inhibits superoxide release induced by fMLP but has not effect on STZ induced superoxide release; moreover, physiological doses of adenosine (10^{-7} to 10^{-6} M) inhibit fMLP-induced superoxide release but not fMLP-induced adhesion. Finally, we have recently found (Carletto et al, manuscript in preparation) that pentoxifylline, a cAMP regulating agent, inhibits superoxide production by blood cells much more effectively than superoxide production by exudate cells suggesting that the latter are more resistant to regulation. Taken together, these evidences strongly suggest that the age-related defect of blood neutrophils described in this paper is due to impairment of the same step that in the transduction pathway of neutrophils is affected by cAMP elevation, and therefore to the activation of protein kinase A and possibly to phospholipase C inhibition (30, 31). The gating by cAMP of signal flow through other pathways in diverse biological processes, including mitogen-activated protein kinases involved in growth and differentiation, has been recently reevaluated (32).

In conclusion our study shows that many PMN functions in elderly subjects, including migration, priming, adhesion, activation by opsonized particles do not differ from young people, suggesting that the overall defence function of these cells is not affected by aging. The only parameter that we have found to be in

marked variance between the two groups is the poor superoxide production after fMLP stimulus of blood cells, a defect from which cells efficiently recover during the migration into exudate. The specificity of this defect in neutrophils from elderly subjects could be a mark of either an increase of cellular cAMP or of an increase of sensitivity of cAMP-related cellular responses. Further studies are necessary to investigate the mechanism of this effect of aging process on cellular functions.

REFERENCES

1. VAN EPPS, D. E., and M. L. GARCIA. 1980. Enhancement of neutrophil function as a result of prior exposure to chemotactic factor. *J. Clin. Invest.* **66:**167–175.
2. BRIHEIM, G., O. STENDHAL, B. I. COBLE, and C. DAHLGREN. 1988. Exudate polymorphonuclear leukocytes isolated from chambers are primed for enhanced response to subsequent stimulation with the chemoattractant fMet-Leu-Phe and C3 opsonized yeast particles. *Inflammation* **12:**141–152.
3. BIASI, D., L. M. BAMBARA, A. CARLETTO, M. CARAFFI, M. C. SERRA, S. CHIRUMBOLO, and P. BELLAVITE. 1993. Factor-specific changes in oxidative burst response of human PMNs in skin window exudates. *Inflammation* **17:**13–23.
4. BIASI, D., L. M. BAMBARA, A. CARLETTO, P. CARAMASCHI, M. C. SERRA, C. SANTONASTASO, P. GUZZO, and P. BELLAVITE. 1993. Molecular mechanisms of priming: exudate human PMNs are primed to fMET-LEU-PHE but have normal intracellular calcium and cAMP responses. *Bull. Mol. Biol. Med.* **18:**225–235.
5. PATY, P. B., R. W. GRAEFF, S. J. MATES, and T. K. HUNT. 1990. Superoxide production by wound PMNs. Evidence for increased activity of the NADPH oxidase. *Arch. Surg.* **125:**65–69.
6. BELLAVITE, P., A. CARLETTO, D. BIASI, P. CARAMASCHI, F. POLI, F. SUTTORA, and L. M. BAMBARA. 1994. Studies of skin window exudate human neutrophils. Complex patterns of adherence to serum-coated surfaces in dependence on fMLP doses. *Inflammation.* **18:**575–587.
7. NURCOMBE, H. L., R. C. BUCKNALL, and S. W. EDWARDS. 1991. Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and action in vivo. *Ann. Rheum. Dis.* **50:**147–153.
8. WARD, P. A., J. S. WARREN, and K. J. JOHNSONN. 1988. Oxygen radicals, inflammation and tissue injury. *Free Rad. Biol. Med.* **5:**403–408.
9. WEISS, S. J. 1989. Tissue destruction by neutrophils. *N. Engl. J. Med.* **320:**365–376.
10. FOLLIN, P., M. P. WYMANN, B. DEWALD, M. CESKA, and C. DAHLGREN. 1991. Human neutrophil migration into skin chambers is associated with production of NAP-1/IL8 and C5a. *Eur. J. Haematol.* **47:**71–76.
11. CORBERAND, J. X., P. F. LAHARRAGUE, and G. FILLOLA. 1986. Neutrophils of healthy aged humans are normal. *Mech. Ageing Dev.* **36:**57–63.
12. ANTONACI, S., E. JIRILLO, M. T. VENTURA, A. R. GAROFALO, and L. BONOMO. 1984. Non-specific immunity in aging: deficiency of monocyte and polymorphonuclear cell-mediated functions. *Mech. Ageing Dev.* **24:**367–375.
13. NIWA, Y., T. KASAMA, Y. MIYACHI, and T. KANOH. 1989. Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. *Life Sci.* **44:**1655–1664.

14. RABATIC, S., A. SABIONCELLO, D. DEKARIS, and I. KARDUN. 1988. Age related changes in functions of peripheral blood phagocytes. *Mech. Ageing Dev.* **45**:223–229.
15. TORTORELLA, C., A. OTTOLENGHI, P. PUGLIESE, E. JIRILLO, and S. ANTONACI. 1993. Relationship between respiratory burst and adhesiveness capacity in elderly polymorphonuclear cells. *Mech. Ageing Dev.* **69**:53–63.
16. NAGEL, J. E., R. S. PYLE, F. J. CHREST, and W. H. ADLER. 1982. Oxidative metabolism and bactericidal capacity of polymorphonuclear leukocyte from young and aged adults. *J. Geronol.* **37**:529–534.
17. LIPSCHITZ, D. A., K. B. UDUPA, and L. A. BOXER. 1988. The role of calcium in the age-related decline of neutrophil function. *Blood* **71**:659–665.
18. LIGTHART, G. J., J. X. CORBERAND, C. FOURNIER, R. GALANAUD, W. HIJMANS, B. KENNES, A. MULLER-HERMELINK, and G. G. STEINMANN. 1984. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mech. Ageing Dev.* **28**:47–55.
19. METCALF, J. A., J. I. GALLIN, W. M. NAUSEEF, and R. K. ROOT. 1986. *In:* Laboratory Manual of Neutrophil Function. Raven Press, New York.
20. SENN, H. J., and W. F. JUNGI. 1975. Neutrophil migration in health and disease. *Semin. Hematol.* **12**:27–45.
21. BABIOR, B. M., R. S. KIPNES, and J. T. CURNUTTE. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**:741–744.
22. BELLAVITE, P., S. CHIRUMBOLO, C. MANSOLDO, G. GANDINI, and P. DRI. 1992. A simultaneous assay for oxidative metabolism and adhesion of human neutrophils. Evidence for correlations and dissociation of the two responses. *J. Leukocyte Biol.* **51**:329–333.
23. MC LAUGHLIN, B., K. O'MALLEY, and T. G. COTTER. 1986. Age-related differences in granulocyte chemotaxis and degranulation. *Clin. Sci.* **70**:59–62.
24. SERLENGA, E., A. OTTOLENGHI, M. T. VENTURA, A. V. POLIGNANO, C. TORTORELLA, E. JIRILLO, and S. ANTONACI. 1990. Monocyte-polymorph- and lymphocyte-mediated chemotactic responsiveness in the elderly. *EOS-Riv. Immunol. Immunopharmacol.* **10**:28–31.
25. RAO, K. M. K., M. S. CURRIE, J. PABMANABHAN, and H. J. COHEN. 1992. Age-related alterations in actin cytoschleton and receptor expression in human leukocytes. *J. Gerontol.* **47**:B37–B44.
26. RIVNAY, B., S. BERGMAN, M. SHINITZKY, and A. GLOBERSON. 1980. Correlation between membrane viscosity, serum cholesterol, lymphocyte activation and ageing in man. *Mech. Ageing Dev.* **12**:119–126.
27. TORTORELLA, C., A. OTTOLENGHI, P. PUGLIESE, E. JIRILLO, and S. ANTONACI. 1994. Polymorphonuclear cells in elderly subjects. *Facts Res. Gerontol.* **3**:211–220.
28. CRONSTEIN, B. N., S. B. KRAMER, G. WEISSMANN, and R. HIRSHHORN. 1983. Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. *J. Exp. Med.* **158**:1160–1177.
29. CRONSTEIN, B. N., L. DAGUMA, D. NICHOLS, A. J. HUTCHISON, and M. WILLIAMS. 1990. The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J. Clin. Invest.* **85**:1150–1157.
30. DELLA BIANCA, V., P. DE TOGNI, M. GRZESKOWIAK, L. M. VICENTINI, and F. DI VIRGILIO. 1986. Cyclic AMP inhibition of phosphoinositide turnover in human neutrophils. *Biochim. Biophys. Acta* **886**:441–447.
31. BELLAVITE, P. 1988. The superoxide-forming enzymatic system of phagotcytes. *Free Radical Biol. Med.* **4**:225–261.
32. IYENGAR, R. 1996. Gating by Cyclic AMP: expanded role for an signaling pathway. *Science* **271**:461–463.

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