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Lymphocyte Adenylate Cyclase and Human Aging[1] (41673)

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Abstract. Adenylate cyclase activity was determined by enzymatic conversion of [^{32}P]ATP to [^{32}P]cAMP using peripheral lymphocytes freshly isolated from human subjects. The lymphocyte enzyme was stimulated by the potent β-adrenergic catecholamine agonist isoproterenol and by the nonhydrolyzable GTP-analog Gpp[NH]p.[3] The two activators had a synergistic effect, and agonist-dependent enzyme activity followed simple Michaelis–Menten kinetics with respect to isoproterenol in the presence but not in the absence of Gpp[NH]p. Cyclic AMP production by intact lymphocytes, determined by protein binding assay, also followed simple Michaelis–Menten kinetics with respect to isoproterenol. K_{act} of isoproterenol was the same in intact cells and the broken cell assay in the presence of Gpp[NH]p, suggesting the indispensable role the GTP-binding coupling factors play in the intact lymphocyte. In 31 human subjects between the age of 21 and 103, adenylate cyclase activity in the presence of isoproterenol, Gpp[NH]p, or isoproterenol in the presence of Gpp[NH]p decreased with the increasing age of the subject. The sensitivity of the enzyme to stimulation by isoproterenol, defined as the K_{act} and determined in the presence of Gpp[NH]p, was the same in lymphocytes from young (<45 years) or elderly (>75 years) subjects. These results suggest a deficiency in the lymphocyte adenylate cyclase system distal to the β-adrenergic catecholamine receptor could account for deterioration of cAMP-mediated components of the immune response which occur with age.

Infirmity generally associated with aging may be the consequence of the deterioration of physiologic mechanisms for maintaining homeostasis. The decline in immune responsiveness that accompanies senescence is one well-known example, and the decrease in target organ sensitivity to β-adrenergic catecholamines is another (1, 2). In lymphoid tissue these may not be mutually exclusive burdens, since β-adrenergic catecholamines probably regulate a variety of immune functions (3). This important class of agonists is believed to function principally, though perhaps not exclusively, through receptor-mediated activation of adenylate cyclase. In fact, we found that isoproterenol-sensitive adenylate cyclase activity of mixed peripheral lymphocytes isolated from aged human subjects was significantly lower than that in cells from younger subjects (4). These enzyme differences could not be accounted for by differences in the distribution of cell types between the two age groups (4). The age-related decrease in human lymphocyte enzyme activity was subsequently confirmed by Abrass and Scarpace (5).

Better understanding of the age related changes in the lymphocyte adenylate cyclase system and its β-adrenergic catecholamine sensitivity could provide important insight into both the causes of aging and ways to alleviate some of its undesirable effects in man. We have determined isoproterenol-dependent β-adrenergic catecholamine activity of lymphocytes from human subjects between the ages of 20 and 103 years. The results indicate a decrease in hormone responsiveness with advancing age.

Materials and Methods. *Human studies and preparation of lymphocytes.* Lymphocytes were isolated from blood collected in heparinized tubes from 31 male and female volunteers between the ages of 20 and 103 years. All subjects were judged disease-free and took no medication. The blood was centrifuged for 20 min at 1000*g* and the resultant buffy coat

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[3] Abbreviations used: Gpp[NH]p, guanosine 5′-[β,-imido]-triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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was resuspended in an equal volume of normal saline. Lymphocytes were isolated from the suspension by using the one-step centrifugation technique of Boyum (6) and commercially available Lymphocyte Separation Medium (Bionetics Laboratory Products, Kensington, Md.). The lymphocyte buffer coat layer was collected, diluted with an equal volume of ice-cold normal saline, and concentrated by centrifugation at 1000*g* for 10 min at 4°C; subsequent steps were carried out at 4°C. Cells were washed 3 times by resuspension in normal saline followed by centrifugation. Cell number was determined in the final saline suspension by hemocytometer count and the washed pellet was resuspended in the appropriate medium at the desired concentration. Cell density was verified by DNA analysis (7) by using highly purified mammalian DNA as standard and 6.4 pg as the DNA content of the human diploid nucleus (8).

Cyclic AMP production by intact cells. Lymphocytes were resuspended at a density of 10^7 cells/ml in Krebs–Ringer bicarbonate buffer containing 5 m*M* theophylline. In the standard assay, incubations at 37°C were begun by the addition of 100 pmole of (−)-isoproterenol in buffer or buffer alone to 10^6 cells (0.1 ml). After 7 min, 0.9 ml of 1-propanol was added to terminate the incubation and the precipitate extracted for 16 hr at room temperature. The insoluble material was concentrated by centrifugation and discarded and the clear supernatant containing cAMP evaporated to dryness in a stream of dry nitrogen. Under these conditions, recovery of [^{3}H]cAMP or exogenous unlabeled cAMP added to determine extraction efficiency was consistently greater than 95%.

Production of cAMP was determined by using the bovine thyroid cAMP-binding protein assay of Orgiazzi *et al.* (9) by dissolving the dried contents of each tube in 1 ml of 0.01 *M* sodium acetate buffer (pH 4.0) with 0.5 m*M* EDTA, 1.0 m*M* theophylline, 0.026 μCi of [^{3}H]cAMP (New England Nuclear, Boston, Mass.; 34.4 Ci/mmole), and binding protein. After 90 min incubation at 4°C, bound was separated from free ligand by the addition of AG1-X2 anion exchange resin according to the modification of Marcus and Orner (10). Results were obtained from a cAMP standard curve extracted and concentrated in an identical manner.

The cAMP production by lymphocytes under these conditions was linear for up to 8 min and unstimulated levels were 3.2 ± 0.4 pmole/10^6 cells.

Adenylate cyclase activity of broken cell preparations. Cells were lysed by the hypotonic conditions of the assay medium. Without further processing, 0.04 ml of the cell suspension was incubated at 37°C in a total volume of 0.15 ml of the assay buffer containing 0.05 *M* Hepes, pH 7.6, 0.001 *M* EGTA, 0.01 *M* $MgCl_2$, 0.01 *M* KCl, 0.001 *M* 3-isobutyl-1-methylxanthine, 0.003 *M* dithiothreitol, 0.25 m*M* ATP (Boehringer Mannheim, Indianapolis, Ind.), 1 μCi [β-^{32}P]ATP (30 Ci/mmole; New England Nuclear Corp., Boston, Mass.), and 60 μg creatine phosphokinase with 310 μg creatine phosphate (Sigma Chemical Co., St. Louis, Mo.) as an ATP-regenerating system. Enzyme activity was determined in triplicate in the basal state (no other additions), in the presence of the GTP-analog guanyl-5′-yl imidodiphosphate (Gpp[NH]p), in the presence of isoproterenol, or isoproterenol with Gpp[NH]p. Ethical consideration precluded collecting blood samples large enough to determine the presence of each additive on the activity of cells from every subject, especially those of advanced age. Consequently properties of the lymphocyte enzyme at each age studied were not necessarily determined using cells from the same subject.

The reaction was started by the addition of the cell suspension and stopped after 5 min by the addition of 0.1 ml of a stopping solution of 1% sodium dodecysulfate, 0.04 *M* ATP, and 0.01 *M* cAMP with 20,000 cpm [^{3}H]cAMP as a recovery standard. The mixture was heated for 3 min at 90°C and cooled, and [^{32}p]cAMP was purified by successive chromatography on columns of Dowex and aluminum oxide according to the procedure of Salomon *et al.* (11). The results, corrected for the recovery of [^{3}H]cAMP (64 ± 7%), were expressed as moles per 1 million cells/5 min. Lymphocyte protein content, determined by the method of Lowry *et al.* (12), was 0.032 ± 0.005 mg/million cells for five separate preparations (mean ± SEM).

Under these conditions, enzyme activity was linear for over 5 min and for cell con-

centrations in excess of 1.5 million per assay tube. Properties of the lymphocyte enzyme are described in greater detail in (4).

Analysis of data. Enzyme activity in the presence of each additive was corrected in every case for activity in its absence. Thus, activity in the presence of isoproterenol or Gpp[NH]p was corrected by subtraction of activity in their absence (basal activity) and activity in the presence of Gpp[NH]p and isoproterenol was corrected by subtraction of activity in the presence of Gpp[NH]p alone. Isoproterenol-dependent enzyme activity was analyzed by Hofstee plot using an unweighted least squares program where simple Michaelis–Menten kinetics prevailed. Analysis of adenylate cyclase activity as a function of subject age was performed with a Hewlett Packard 9830A computer using a polynomial regression program.

Results. The potent β-adrenergic catecholamine agonist isoproterenol stimulated cAMP production by intact lymphocytes over twofold (Fig. 1). The inset in Fig. 1 shows that when the concentration-dependent effect of isoproterenol was analyzed over its stimulatory range

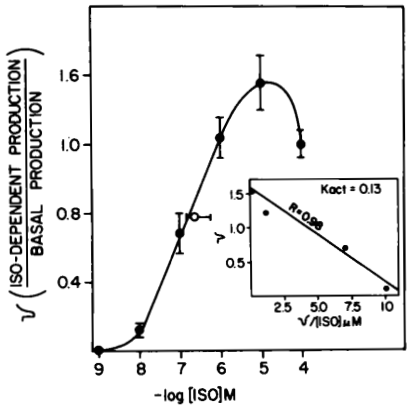

FIG. 1. Isoproterenol dose-dependent cAMP production by intact lymphocytes. Freshly isolated cells were incubated with agonist at the indicated concentration (ISO) in RPMI 1640 and cAMP determined as described in Materials and Methods. The agonist-dependent activity of cells from three separate young (<45 years) subjects (closed circles, mean ± SD) was measured and analyzed by Hofstee plot (inset). The mean (±SD) K_{act} of the three preparations, the negative slope of the Hofstee plots (correlation coefficient—R) is plotted at one-half V_{max} (open circle), also determined from the linear plot. Isoproterenol-dependent cAMP production was 5.45 pmole/1 million cells.

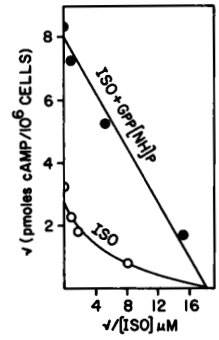

FIG. 2. Effect of exogenous guanyl nucleotide on isoproterenol-dependent adenylate cyclase activity of the broken cell preparation. Isoproterenol (ISO) dose-dependent enzyme activity was determined in the absence (open circles) or presence (closed circles) of 300 μM Gpp[NH]p as described in Materials and Methods and analyzed by Hofstee plot. The figure, the results of characterization of one preparation, is typical of those obtained with six separate preparations.

by Hofstee plot, hormone-dependent cAMP production followed simple first-order kinetics with respect to the agonist as reflected by the linearity of the plot. The activation constant (K_{act}), the negative slope of the Hofstee plot, was 0.13 μM (Fig. 1).

The hypotonic conditions of the broken cell adenylate cyclase assay lysed the cells and the nonhydrolyzable GTP analog guanyl-5′-yl imidodiphosphate (Gpp[NH]p) as well as isoproterenol stimulated the enzyme (4). Figure 2 is representative of the synergistic effect the two activators had on adenylate cyclase. More product ([^{32}P]cAMP) was formed in response to isoproterenol in the presence of Gpp[NH]p than in its absence. Moreover, isoproterenol-dependent adenylate cyclase activation followed first-order kinetics with respect to the agonist in the presence of a saturating (300 μM) concentration of Gpp[NH]p. These guanyl nucleotide effects reflect the role of the GTP-binding coupling factor in hormone stimulation of lymphocyte adenylate cyclase. In addition, isoproterenol-dependent cAMP production by the broken cell preparation resembled that in the intact cells (with respect to first-order kinetics) only in the presence of the guanyl nucleotide.

Isoproterenol-dependent adenylate cyclase activity determined in the broken cell enzyme assay in both the absence and presence of a

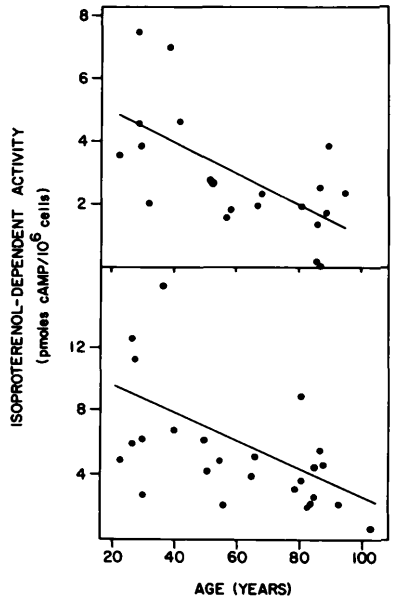

FIG. 3. Effect of subject age on agonist-dependent adenylate cyclase activity. Enzyme activity was measured in the broken cell assay in the absence of Gpp[NH]p in (upper panel) as described in Materials and Methods in lymphocytes from 22 subjects and in the presence of 300 μM Gpp[NH]p in cells from 25 subjects (lower panel). The slopes, determined as described in the text, were both significantly different ($P < 0.03$) from zero with correlation coefficients of 0.60 (upper) and 0.55 (lower).

saturating (100 μM) concentration of Gpp[NH]p is shown as a function of subject age in Fig. 3. As age increased, there was a significant decrease in hormone-dependent enzyme activity. When the age-related loss of activity was analyzed by polynomial regression up to a power of 6, the points were best fit by a straight line ($R > 0.55$) with a slope significantly ($P < 0.03$) different from zero whether activity had been determined in the absence (Fig. 3, top panel) or presence (Fig. 3, bottom panel) of Gpp[NH]p.

These results could be accounted for by either an age-related decrease in isoproterenol sensitivity of the enzyme (K_{act}), or a decrease in the activity of the enzyme (V_{max}). We distinguished between these possibilities by assaying lymphocytes from young (<35 years) and aged (>65 years) subjects for isoproterenol dose-dependent adenylate cyclase activity in the presence of Gpp[NH]p, conditions under which the rate of cAMP formation resembled that by the intact cells. Figure 4 (open symbols) shows there was no difference in K_{act} of isoproterenol-dependent adenylate cyclase activity of lymphocytes from the two age groups, and that isoproterenol doses as great as 300 μM (more than 2500 times that of K_{act}) failed to stimulate the enzyme activity of "aged" cells to levels found in "young" cells. The inset in Fig. 4 shows that Gpp[NH]p responsiveness, like isoproterenol responsiveness, was significantly decreased as a function of the age of the subject from which the lymphocytes were isolated. The values, also analyzed to a power of 6 by polynomial regression, were best described by a straight line ($R > 0.51$) whose slope was significantly different from zero ($P < 0.02$).

Discussion. Adenylate cyclase activity of freshly isolated human lymphocytes decreased as a function of age in a sample of 31 subjects between the ages of 20 and 103 years. The loss of activity associated with aging included

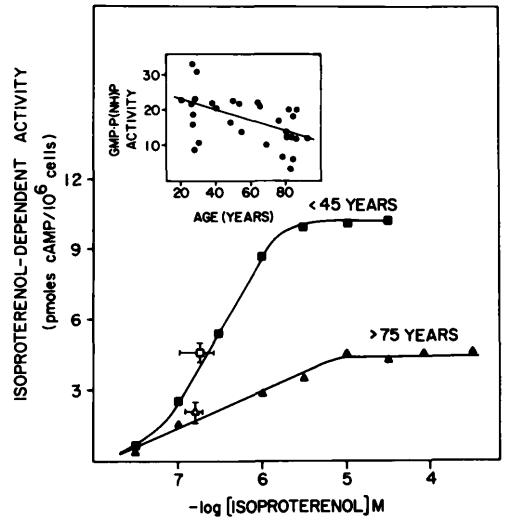

FIG. 4. Effect of subject age on K_{act} for isoproterenol. Isoproterenol dose-dependent adenylate cyclase activity was determined in the broken cell assay in the presence of 300 μM Gpp[NH]p using lymphocytes from three young or three aged subjects. K_{act} and one-half V_{max} were determined by Hofstee plot and are plotted (open symbols) as the mean ± SD of the three separate experiments. Inset, Gpp[NH]p-dependent enzyme activity (picomoles cAMP/1 million cells) of lymphocytes from 30 subjects plotted as a function of their age. The slope, determined as described in the text, was significantly different ($P < 0.02$) from zero.

isoproterenol-dependent enzyme activity, i.e., the potent β-adrenergic catecholamine agonist failed to restore the lower activity of cells from aged subjects to levels approaching that of cells from younger subjects. The loss of hormone-dependent enzyme activity was not restored by the GTP-analog Gpp[NH]p, a significant observation since it was in the presence of the guanyl nucleotide that the kinetics of isoproterenol-dependent product (cAMP) formation in the broken cell preparation resembled that of intact cells. The requirement was obvious from the effect the addition of exogenous guanyl nucleotide (Gpp[NH]p) had on the K_{act} of the broken cell enzyme. The absence of a significant difference between the K_{act} of isoproterenol in intact cells and in the broken cell assay in the presence of Gpp[NH]p implies but does not prove the importance of the guanyl nucleotides and GTP-binding coupling factors in β-adrenergic catecholamine regulated functions of the intact lymphocyte. Their importance in coupling receptors with adenylate cyclase catalytic subunits in cell-free enzyme systems is well-known (13).

In light of its effects on immune function, there has been appreciable interest in the effects of human aging on lymphocyte adenylate cyclase activity, but with ambiguous results. Kraft and Castleden (14) measured cAMP production in response to isoproterenol in intact lymphocytes from groups of young (<30 years) and aged (>68 years) human subjects. They found no difference in either basal or agonist-dependent rate, but the variance within the age groups was substantial. Tam and Walford (15) have described age-related changes in cAMP production by peripheral human T lymphocytes. Although the basal rate of production by intact cells decreased dramatically with subject age, the specific activity of adenylate cyclase in the broken cell assay increased with age. Only basal enzyme activity was determined, however, and this was reported as 0.27 $pmol/mg \cdot min^{-1}$ (mean ± SEM). The large disparity between lymphocyte adenylate cyclase activity determined by Tam and Walford (15) and by us is not explained by differences in the conditions of the enzyme assay, which were similar, or differences between the rate of cAMP production by B compared with T lymphocytes (4, 16). One possibility is that procedures used to fractionate lymphocytes producted purified cells in which adenylate cyclase was particularly labile to the deleterious effects of homogenization. In contrast, we used a gentle procedure which produced broken cell preparations of K_{act} for isoproterenol in the presence of Gpp[NH]p which was the same as that of intact cells (0.11 as opposed to 0.13 μM), suggesting the coupling mechanism was largely preserved. In any event, our results suggest the age-related decrease in the rate of cAMP production by intact cells reported by TAM and Walford (15) are due to changes at the adenylate cyclase enzyme level.

The reasons adenylate cyclase activity decreases with subject age are obscure. The decrease in enzyme activity in the presence of isoproterenol and Gpp[NH]p with subject age was marked when corrected by subtraction for activity without the additions. This suggests the rate of decline in hormone responsiveness was somewhat specific and greater than that of the adenylate cyclase catalytic subunits. We detected no difference in the distribution of cell types in our lymphocyte preparations from the different age groups (4). Moreover, the results reported in Ref. (17), which suggested but did not prove that the decrease in activity associated with age could be reversed *in vitro,* cannot be attributed to age-related changes in cell subpopulations.

Although senescence was associated with elevations in plasma catecholamine levels, there were important qualitative differences between the adenylate cyclase activity of lymphocytes from the elderly and that of cells desensitized by exposure to catecholamines (4). The age-related decrease in responsiveness to Gpp[NH]p as well as isoproterenol suggests important changes occur distal to the β-adrenergic catecholamine receptor. Absence of an effect of age on the K_{act} by isoproterenol suggest no changes occur in the lymphocyte concentration of receptors. Abrass and Scarpace (18) measured β-adrenergic catecholamine receptors in lymphocytes from young and aged human subjects by radioactive antagonist (dihydro[^{3}H]alprenolol) specific binding methods and reported no difference between the age groups, although the differences between individuals were large.

Our results further indicate a component of the lymphocyte β-adrenergic catecholamine

sensitive adenylate cyclase system distal to the receptor change with advancing age in man. The possible restorative effect factors from lymphocytes from young subjects had on adenylate cyclase activity of lymphocytes from aged subjects which we described previously suggest these changes included a decrease in the concentration of catalytic subunits or the guanyl nucleotide-binding coupling factors (17). Cumulatively these changes might account, in part, for not only some age-related deterioration in immune function in man, but also for decreases in β-adrenergic catecholamine sensitivity of target tissues less accessible than the lymphocyte.

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1. Guarnieri T, Filburn CR, Zitnik G, Roth GS, Lakatta EG. Contractile and biochemical correlates of β-adrenergic stimulation of the aged heart. Amer J Physiol. **239**:H501–H508, 1980.
2. Vestal RE, Wood AJJ, Shand DG. Reduced β-adrenoreceptor sensitivity in the elderly. Clin Pharmacol Ther **26**:181–186, 1979.
3. Bourne HR, Lichtenstein LM, Melmon KL, Henney CS, Weinstein Y, Shearer GM. Modulation of inflammation and immunity by cyclic AMP. Science **184**:427–429, 1974.
4. Krall JF, Connelly M, Weisbart R, Tuck ML. Age-related elevation of plasma catecholamine concentration and reduced responsiveness of lymphocyte adenylate cyclase. J Clin Endocrinol Metabol **52**:863–867, 1981.
5. Abrass IB, Scarpace PJ. Catalytic activity of adenylate cyclase: Reduced activity in aged human lymphocytes. J Clin Endocrinol Metab **55**:1026–1028, 1982.
6. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest **21**(Suppl 97):77–89, 1968.
7. Burton K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorametric estimation of DNA. Biochem. J **62**:315–322, 1956.
8. Sober HA. Handbook of Biochemistry. Cleveland, Chem Rubber Co, pH-112, 1970.
9. Orgiazzi J, Chopra IJ, Williams DE, Solomon DH. Evidence for normal thyroidal adenyl cyclase, cAMP, and protein kinase activity in Graves disease. J Clin Endocrinol Metabol **40**:248–252, 1975.
10. Marcus R, Orner E. Cyclic AMP production in infant rat calvaria *in vitro:* Interaction of prostaglandins with parathyroid hormone. Endocrinology **101**:1570–1578, 1977.
11. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. Anal Biochem. **58**:541–598, 1974.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **195**:265–275, 1951.
13. Ross EM, Gilman AG. Biochemical properties of hormone-sensitive adenylate cyclase. Annu Rev Biochem. **49**:533–564, 1980.
14. Kraft CA, Castledan CM. The effect of aging on β-adrenoreceptor-stimulated cAMP formation in human lymphocytes. Clin Sci **60**:587–589, 1981.
15. Tam CF, Walford RL. Alterations in cyclic nucleotides and cyclase-specific activities in T-lymphocytes of aging normal humans and patients with Down's syndrome. J Immunol **125**:1556–1670, 1980.
16. Bishopric NH, Cohen HJ, Lefkowitz RJ. Beta-adrenergic receptors in lymphocyte subpopulations. J Allergy Clin Immunol **65**:29–33, 1980.
17. Krall JF, Connelly M, Tuck ML. Evidence for reversibility of age related decrease in human lymphocyte adenylate cyclase activity. Biochem Biophys Res Commun **99**:1028–1034, 1981.
18. Abrass IB, Scarpace PJ. Human lymphocyte beta-adrenergic receptors are unaltered with age. J Gerontol **36**:298–301, 1981.

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