Restoration of Immune Responses of Aging Hamsters by Treatment with Isoprinosine

KWONG Y. TSANG, H. HUGH FUDENBERG, and MARK J. GNAGY, *Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina 29425*

ABSTRACT Immune competence declines with advanced age in hamsters, as in other laboratory mammals and in humans. We found significant alterations in the functional parameters of different populations of immunocytes (natural killer cells, T cells, monocytes, and suppressor cells) in aging hamsters, beginning at ~14 mo of age. Natural killer cytotoxicity, phytohemagglutinin-induced lymphocyte stimulation, and monocyte chemotaxis were decreased in aging Lak:LvG(Syr) outbred hamsters. When old hamsters were given a single injection (5 mg/kg body wt) of isoprinosine, a chemical immune potentiator, these three immune parameters increased almost to the levels found in young adult hamsters but returned to pretreatment levels after 7 d. Suppressor cell activity for the lymphocyte response to phytohemagglutinin, which increased with age, was decreased after treatment. In old hamsters treated with weekly injections of isoprinosine, these four immunological parameters remained at or near the levels found in young adults.

INTRODUCTION

Various immunological functions and responses are affected by the aging process. In humans, responsiveness to thymus-dependent antigens originally contacted in early life decreases with advancing age (1, 2). T cell functions, such as primary delayed skin reaction in response to antigens to which individuals have not been sensitized previously (e.g., dinitrochlorobenzene) and the proliferative capacity of T cells in response to phytohemagglutinin (PHA),[1] also decline with age (3–8). The data of Girard et al. (9) regarding cell-mediated immunity in 880 hospitalized patients indicated a decrease in the absolute number of circulating T lymphocytes, and also functional impairment of T cells.

In mice, T cell-dependent functions, such as the lymphocyte response to PHA in vitro, decline with age (10–14); resistance to challenge with syngeneic and allogeneic tumor cells in vivo is also dramatically decreased in old animals (15, 16). Decreased absolute numbers of lymphocytes in peripheral blood in aging hamsters have also been reported (17). We have previously found that immune responses such as monocyte chemotaxis, lymphocyte response to PHA stimulation, and natural killer (NK) activity are decreased, and that suppressor cell activity is increased in aging hamsters (18).

Only a few chemical agents possess immunorestorative activity. These include double-stranded polynucleotides (19, 20), thymic hormones (21, 22), certain free radical inhibitors such as vitamin E and coenzyme Q_{10} (23, 24), levamisole (25, 26), and mercaptoethanol (27, 28). Recently, isoprinosine (ISO), the *p*-acetamidobenzoic acid salt of *N*,*N*-dimethylamino-2-propanol:inosine complex (3:1 molar ratio) has been shown to be an effective immune potentiator (29, 30). Wybran (31) has demonstrated that ISO can enhance the PHA response of lymphocytes from cancer patients, and Lesourd et al. (32) have reported that it can restore cell-mediated immunity in patients with acute encephalitis. In an animal model of human osteosarcoma, we have shown that ISO can augment proliferation of peripheral blood lymphocytes from tumor-bearing animals in vitro and can increase the functional capacity of their immune system (33). The present investigation was designed to examine the effects of ISO on the immune responses of aging hamsters.

METHODS

Animals. Outbred hamsters, strain Lak:LvG (Syr), were used in this investigation. They were divided into eight

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[1] *Abbreviations used in this paper:* FCI, functional chemotactic index; ISO, isoprinosine; LS, lymphocyte stimulation; MC, monocyte chemotaxis; NK, natural killer; PHA, phytohemagglutinin; SC, suppressor cell; TFS, thymosin.

groups according to age (0.5, 2, 4, 8, 12, 14, 18, and 24 mo). ISO, supplied by Newport Pharmaceuticals International (Newport Beach, CA), was freshly dissolved in saline and injected intraperitoneally at a concentration of 5 mg/kg body wt, the optimal concentration indicated in our previous study (33). Hamsters were bled by cardiac puncture and lymphocytes were isolated on a Ficoll-Hypaque gradient as described by Boyum (34).

Lymphocyte stimulation (LS). A modification of the method described by Ron et al. (35) was used for assay of PHA-induced LS. 1×10^5 cells in 0.1 ml RPMI 1640 medium with 10% fetal calf serum were inoculated in the wells of a 96-well flat-bottomed microtest plate and PHA (Wellcome Reagents) was added at 10 μg/well. Each assay was performed in triplicate. The lymphocyte cultures were incubated for 48 h at 37°C in a 5% CO_2 incubator, 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL, 5 Ci/mmol) was added to each well, and incubation was continued for 24 h. The cultures were harvested in a Mash I cell harvester (Microbiological Associates, Walkersville, MD).

NK cell activity. For measurement of NK activity, Raji cells were used as target cells in an assay as described previously for hamster NK activity (36). Effector cells were prepared from peripheral blood lymphocytes and Ficoll-Hypaque-isolated peripheral blood (37). Mononuclear cells were depleted of adherent cells by incubation of the mononuclear cells for 1 h at 37°C on 60-mm plastic culture dishes. The purified mononuclear cells contained <0.5% monocytes based on morphology and nonspecific esterase staining. 100 μl of nonadherent cells were added with 100 μl of ^{51}Cr-labeled target cells (1×10^4) in the wells of a flat-bottomed microtest plate at a ratio of 50:1 or 25:1. Target cells incubated without effector cells were used to determine the level of spontaneous lysis. Maximum lysis was determined by adding 10% Triton X-100 solution (no effector cells) instead of medium to the well. The percentage cytotoxicity was calculated as: Percent cytotoxicity = mean cpm test − mean cpm spontaneous lysis/mean cpm maximum lysis − mean cpm spontaneous lysis × 100. Each assay was performed in triplicate.

Chemotaxis. The method of Tsang et al. (38) was used to measure monocyte chemotaxis (MC). Chemotactic factor was generated in fresh hamster serum by incubation with 1 mg *Salmonella typhosa* 0901 endotoxin (Difco Laboratories, Inc., Detroit, MI) per ml of serum at 37°C for 1 h followed by 56°C for 30 min. In Boyden chemotaxis chambers, monocytes were separated from the stimulus with a 5-μm pore size polycarbonate filter. The chambers were incubated at 37°C in a 5% CO_2 incubator for 3 h. The functional chemotactic index (FCI) was calculated by the following formula: FCI = number of migrating cells in experimental chamber/number of migrating cells in control chamber × 100. Each assay was performed in triplicate.

For the study of the effects of ISO on macrophage chemotaxis in vitro, the following procedures were used. Only adherent cells were used in this assay. Macrophages were incubated with ISO at an optimal concentration (100 μg/10^6 cells per ml) for 2 h at room temperature. At the end of the incubation time, ISO-treated macrophages were washed and used in the assay. Macrophages incubated in medium alone were used as controls.

Suppressor cell activity. The method described by Padarathsingh et al. (39) was used to assay suppressor cell activity (SC). Mononuclear cells were isolated on a Ficoll-Hypaque gradient. PHA was used as mitogen at a concentration of 10 μg/well with mononuclear cells cultured at a concentration of 2×10^5 cells/well in microtest plates. For co-cultures 1×10^5 cells collected from old hamsters (18 mo of age) before and after ISO treatment were mixed with 1×10^5 cells from young hamsters (3–4 mo of age). The cultures were incubated at 37°C in a 5% CO_2 incubator for 3 d, and 1 μCi of [^{3}H]thymidine was added to each well 24 h before the cultures were harvested. Mononuclear cell cultures from young hamsters or old hamsters alone were used as controls. Percentage suppression was calculated by the following formula: Percent suppression = [1 − (cpm $Y + O$)/(cpm Y)] × 100. Each experiment was performed in triplicate.

RESULTS

The effects of age on LS, NK, and monocyte chemotaxis (MC) activities are shown in Table I. Each age group contained 15 hamsters (five groups were used for each determination, three hamsters in each group). The highest responses in the three assays were observed in the 8-mo-old group. The 18- and 24-mo-old groups had the lowest responses. The LS response to PHA was significantly decreased in 12-, 14-, 18- and 24-mo-old hamsters as compared wth the younger age groups ($P < 0.005$ by Student's t test). NK cytotoxicity was significantly decreased in the 14-, 18-, and 24-mo-old groups as compared to 4-, 8-, and 12-mo-old groups ($P < 0.005$). Monocyte chemotaxis was also depressed significantly in the 14-, 18-, and 24-mo-old groups as compared to the younger age groups ($P < 0.01$).

The effects of ISO on macrophage chemotaxis in hamsters of various ages are shown in Table II. Significant increases in macrophage chemotaxis activities were observed in hamsters of 12, 14, and 18 mo of age when treated with ISO in vitro. No significant increases in macrophage chemotaxis activities were seen in ham-

TABLE I
Immune Responses in Hamsters of Various Age Groups

Age	PHA-induced lymphocyte proliferation	NK cell activity	MC
mo	*mean cpm/10^4±SE*	*mean % lysis±SE*	*FCI*
0.5	8,178±114.6	4.5±1.8‡	85.0
2.0	7,972±156.5	1.2±4.8‡	91.8
4.0	8,467±196.5	47.3±7.2	86.3
8.0	9,174±198.7	54.2±6.5	100.0
12.0	4,354±106.4*	52.6±5.9	65.5§
14.0	4,106±117.4*	21.4±3.8‡	43.3§
18.0	2,316±98.6*	14.1±3.1‡	37.6§
24.0	2,410±79.8*	12.9±4.8‡	36.8§

Five groups of hamsters were used for each determination, each group consisting of three hamsters. Student's t test was used for all statistical analysis.
* Statistical significance, $P < 0.005$.
‡ $P < 0.01$.
§ $P < 0.01$.

TABLE II
*Effects of ISO on MC in Hamsters In Vitro**

Age	Without ISO	With ISO	*P*
mo			
0.5	82.6±2.51	85.3±4.34	NS
2.0	88.3±2.08	91.3±3.21	NS
4.0	86.3±4.04	91.0±1.00	NS
8.0	100.0±0.57	97.0±3.00	NS
12.0	61.0±2.64	80.6±2.08	$P < 0.005$
14.0	44.6±4.50	66.0±8.18	$P < 0.01$
18.0	34.3±4.04	56.3±5.50	$P < 0.01$

* Results are expressed as FCI. Three groups of hamsters were used for each determination, each group consisting of five hamsters. Student's *t* test was used for all statistical analyses.

sters of 0.5, 2, 4, and 8 mo of age after ISO treatment in vitro.

The effects of ISO injections on the LS responses to PHA are shown in Fig. 1a. Three age groups were used (14, 18, and 24 mo old) with 15 hamsters in each group. Increases in LS were seen on days 1, 3, and 5 postinjection, returning to control levels on day 7. Fig. 1b shows the effects of ISO on NK cytotoxicity in three groups of hamsters (14, 18, and 24 mo old), each containing 15 hamsters, and Fig. 1c shows the results of MC assays in three groups of 9 hamsters each. Enhancement of both NK and MC activity was seen on days 1, 3, and 5 after ISO injection, and both returned to control levels on day 7 in all three age groups.

The number of macrophages in the mononuclear cell preparations of young hamsters (3–4 mo old) and old hamsters (18 mo old) was determined by the nonspecific esterase staining method. There were no significant differences in the percentage of macrophages between the young (5–7.5%) and old hamsters (6–8.4%). The effects of ISO on SC activity in aging hamsters are shown in Table III. 10 18-mo-old hamsters were used in this study. Lymphocytes from young hamsters were

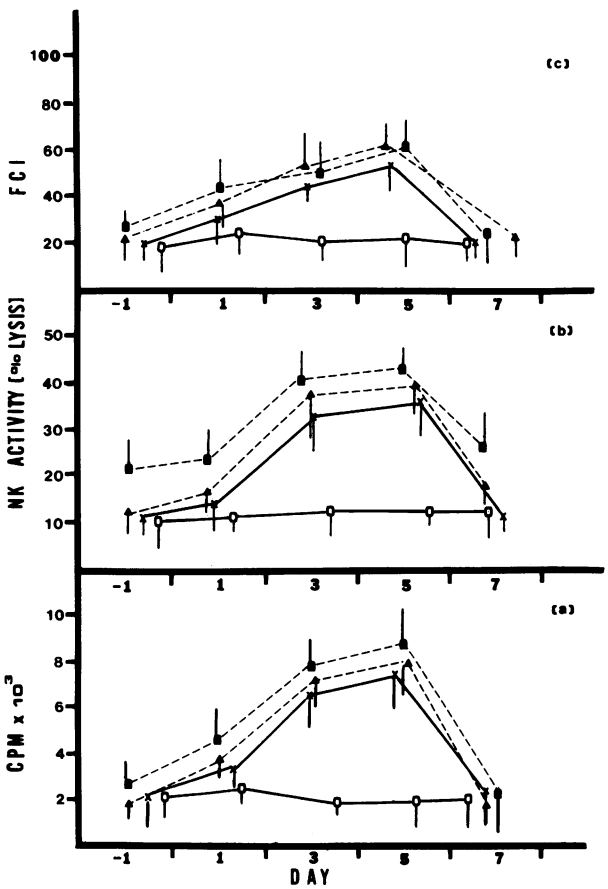

FIGURE 1 Effects of single injection of ISO on (a) PHA-induced lymphocyte proliferation, (b) NK activity, and (c) MC in aging hamsters: (○) 14 mo, (▲) 18 mo, (×) 24 mo, (○) control (14 mo old).

TABLE III
Effects of ISO on SC Activity in Aging Hamsters

Culture* Y O	Before ISO treatment cpm‡	Before ISO treatment % suppression§	After ISO treatment cpm‡	After ISO treatment % suppression§
1	6,349±593	—	7,996±449	—
1 + 1	1,097±69	82.8	7,814±560	3.0
1 + 2	1,541±87	75.8	8,062±671	0
1 + 3	1,112±98	82.5	7,094±698	1.2
1 + 4	987±56	84.5	7,126±709	11.0
1 + 5	1,610±107	74.7	7,012±604	12.4
1 + 6	1,812±124	71.5	6,984±587	12.7
1 + 7	1,937±94	69.5	5,837±439	27.1
1 + 8	1,406±119	77.9	6,019±672	24.8
1 + 9	2,013±143	68.3	7,504±704	6.2
1 + 10	2,436±109	61.7	8,126±568	0
2	9,871±746	—	8,964±765	—
2 + 1	1,916±114	80.6	8,166±643	9.0
2 + 2	1,314±134	86.7	8,431±747	6.0
2 + 3	1,659±156	83.2	7,561±564	16.0
2 + 4	1,927±148	80.5	7,844±766	13.0
2 + 5	1,762±114	82.2	7,124±567	20.6
2 + 6	1,845±125	81.6	7,344±671	18.1
2 + 7	1,978±116	80.0	8,126±562	9.4
2 + 8	2,042±187	79.4	6,021±413	32.9
2 + 9	2,137±104	78.4	6,124±409	31.7
2 + 10	1,534±109	84.5	6,849±514	23.6

* Lymphocytes from 18-mo-old hamsters (O_1–O_{10}) were co-cultured with lymphocytes from the pooled blood of two different groups of three 4-mo-old hamsters (Y_1 and Y_2).
‡ Mean±SE for three determinations.
§ % suppression = [1 − (cpm Y + O)/(cpm Y)] × 100.
Mean percent suppression to Y_1 were 74.9 (before ISO treatment) and 9.84 (after ISO treatment). Mean percent suppression to Y_2 were 81.71 (before ISO treatment) and 17.2 (after ISO treatment).

obtained from the pooled blood of two different groups of three 4-mo-old hamsters. Assays were performed before and on the 4th d after intraperitoneal injection of ISO in the old hamsters. SC activity for PHA-induced lymphocyte proliferation was significantly reduced in all the old hamsters treated with ISO ($P < 0.005$).

The effects of continuous treatment with ISO on the LS, NK, and MC activities of aging hamsters are shown in Fig. 2, and SC activity is shown in Fig. 3. ISO was injected intraperitoneally once per week, beginning at the 14th mo for the LS, NK, and MC studies and at the 18th mo for the SC study, and blood was drawn for assays once per month on the day before the next injection of ISO. All four immune responses, which were significantly decreased in the untreated group, were normal or near normal (as compared to young adult hamsters) in the ISO-treated group of aging hamsters.

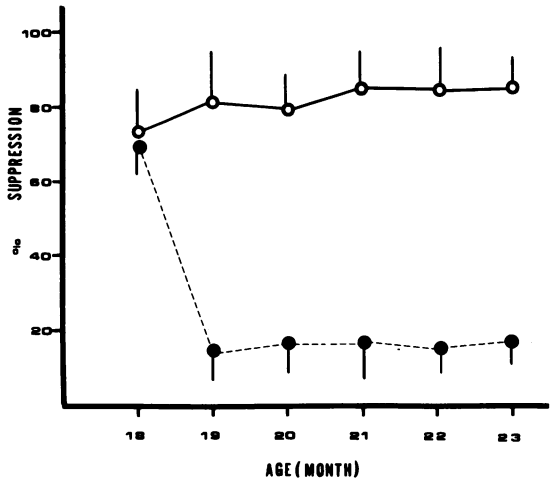

FIGURE 3 Effects of weekly injections of ISO on suppressor cell activity in aging hamsters. SC assays were performed before and after the beginning of ISO injections. (●) ISO-treated hamsters, 18 mo old at the beginning of treatment; (○) age-matched controls.

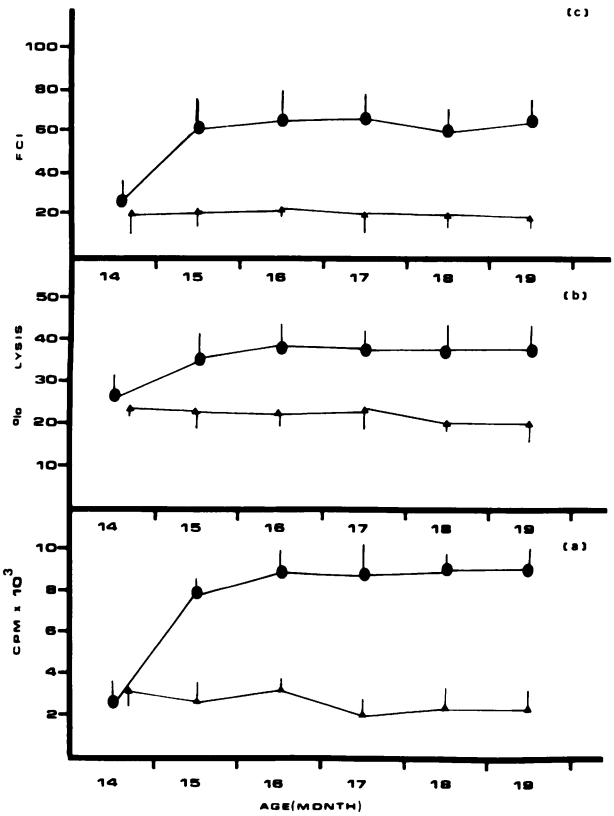

FIGURE 2 Effects of weekly injections of ISO on (a) PHA-induced lymphocyte proliferation, (b) NK activity, and (c) MC in aging hamsters. Immune assays were performed before and after the beginning of ISO injections. (●) ISO-treated hamsters, 14 mo old at the beginning of treatment; (▲) age-matched controls.

DISCUSSION

The results of our investigation in hamsters confirm many findings that cell-mediated immune activity declines with age in mammals (3, 4, 5, 18). LS, NK, and MC activities were significantly decreased and SC activity was significantly elevated in hamsters in the age group of 12–14 mo or older. The present investigation also suggests that ISO can be used to restore or maintain normal immune function in aging animals.

Prostaglandins of the E series have been shown to have inhibitory effects on NK cell activity (40, 41). To eliminate the possibility of inhibition of NK activity by prostaglandins, we used nonadherent cells as effector cells in our assay. Our effector cell preparation contained <0.5% monocytes.

In vitro effects of ISO on macrophage chemotaxis were studied in hamsters of various ages. Our data show that ISO increased MC activity in hamsters 12, 14, and 18 mo old. These hamsters had low MC activities without ISO treatment. However, treatment of macrophages with ISO in vitro did not increase the MC activity in hamsters 0.5, 2, 4, and 8 mo old that had normal or close to normal activity. These results were similar to the results of our study in humans (37).

There were no significant differences in the percent of macrophage contents between the young and old hamsters of the strain we studied. For this reason, we believe that the suppression in PHA-stimulated lymphocyte proliferation in old hamsters was not caused by the difference in the number of macrophages in the cell preparation.

The decline in immune activity in aging animals could result from changes in the immune cells, changes in their milieu, or both. Studies by Makinodan and coworkers (42–46) have suggested that although both types of changes affect the immune response in mice, much of the normal age-related decline in immunity can be attributed to changes in the immune cells. These changes may be in the number of T lymphocytes (49), the capacity of lymphocytes to function (48, 49), and/or alterations in subpopulations of lymphocytes (50).

We attempted to restore the declining immune functions of aging hamsters by administering ISO. We found that a single injection of ISO elevated their LS, NK, and MC activities, with the peak activity on day 5 postinjection as found previously (33), and that continuous injections of ISO restored the LS, NK, MC, and SC activities of aging hamsters to the levels of young adult hamsters. Several studies of other immune potentiators, such as thymic hormones and levamisole, in aging have also been reported. For example, Cowan et al. (51) found that mixed lymphocyte reactivity was significantly increased when thymosin (TF5) was incubated with lymphocytes from elderly individuals. Levamisole, which does not increase immune functions in vitro, is effective only when administered in vivo over an extended period (52). This would suggest that levamisole itself may not exert its enhancing effect directly on the immune cells, but rather through either another cell type or a metabolite. In contrast, ISO can restore immune function both in vivo and in vitro. The mechanism of action of ISO in restoring the immune functions of aging hamsters is not known, but it may be mediated partly by factors such as interleukin-2 (53) or interleukin-1 (54) and our data suggest that it acts on both T cells and monocytes. Further investigations have been undertaken to study the effects of ISO on life span, susceptibility to disease, and tumor development in aging hamsters. The information obtained from these studies may be valuable in developing new methods for delaying the onset, decreasing the rate, or reversing the effects of declining immune function in aging animals.

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