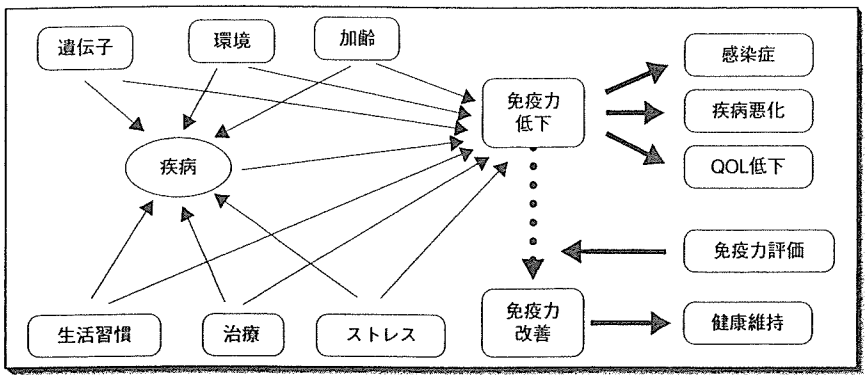

図1．免疫力を低下させる因子
加齢（老化）に加えて，ストレス，環境，遺伝的背景，生活習慣などが免疫機能低下の要因となる。さらに疾病とそれに対する治療が免疫力を低下させる因子となることを忘れてはならない。免疫力の低下は感染症にかかりやすい状態を作り，QOLの低下，そしてすでにかかっている疾病がさらに悪化する要因となる。免疫力の低下を適切に評価し，免疫力を改善できれば，健康を維持させることが可能となる。

下し始める。すなわち，20歳を過ぎると免疫機能は早くも低下し始め，40歳代でピークの50%，70歳代で10%前後に低下することもある[1)2)]。低下の程度は大きな個人差があるのが特徴で，長寿の人は免疫機能が高く維持されていることもわかってきた。したがって，免疫機能を評価するときは，このリンパ球系の機能が中心となる。

免疫力が低下するとどうなるか

繰り返すことになるが，免疫力は感染症に対抗するだけでなく，身体の中の血液，体液，組織内の恒常性を保つ上で（ホメオスターシス），大きな役割を果たしている。したがって，免疫力が低下すると，感染症にかかりやすくなるだけでなく，ストレスへの対抗力が低下し，身体に違和感を感じることが多くなる。いわゆる体力というものは，筋力と誤解されやすいが，その本質は感染症やストレスに対抗する能力であり，免疫力の総合的な能力が大きな役割を占めている。したがって，健康維持のためには，免疫力のレベルを知ることが重要であり，その程度を測定することが必要となる。

免疫系の機能の測定

感染症に対抗する身体のシステムは免疫系であることは誰でも知っているが，自分の免疫系の能力・レベルを知っている人は少ない。血圧，血糖値，コレステロール値など，健康の指標となる検査データはたくさんある。しかし，免疫系の機能に関するデータは測定することも少なく，知らない人が大部分である。

免疫系の機能測定で問題になるのは，多彩な機能をもつリンパ球からなる獲得免疫系の能力である。一般的には，免疫力と概念的にいわれているが，その能力を一つの免疫機能やマーカーで表現することは難しい。我々はその総合的な免疫力を10項目の免疫マーカーや機能を測定し，その一つ一つについて，データベースに基づいて3段階のスコアを与え，10項目のスコアの総和でもって，総合的な免疫力の指標（SIV）とする方法を開発した。スコアの総和は10～30になるが，その分布を健常人と癌患者について比べてみると，このSIVの意味が少しわかる（図2）。そのSIVをグレード1～5の5段階に分けて表示するとよりわかりやすくなる。図3に示すように，癌患者の免疫力のグレードは低いところに分布していることが一目瞭然である。このことにより，誰でも自分の免疫機能がどの程度であるか，5段階表示で簡単に認識できるようになった。

免疫機能の回復方法

免疫力を評価して，低下があれば，回復を考える必要がある。特に高齢者が増加の一途をたどる現在においては，この免疫機能の回復は抗感染力の増強とともにQOLの改善につながるので，極めて大事である。以下に免疫機能の回復方法について概説する[1)2)4)5)]。

1．既存する免疫系細胞の機能の亢進と回復

①栄養調節，②抗酸化物質投与，③ホルモン投与，④低用量の放射線や抗癌剤，⑤ワクチン，⑥漢方薬補剤，⑦抗原の経口投与，⑧サイトカイン治療，⑨運動

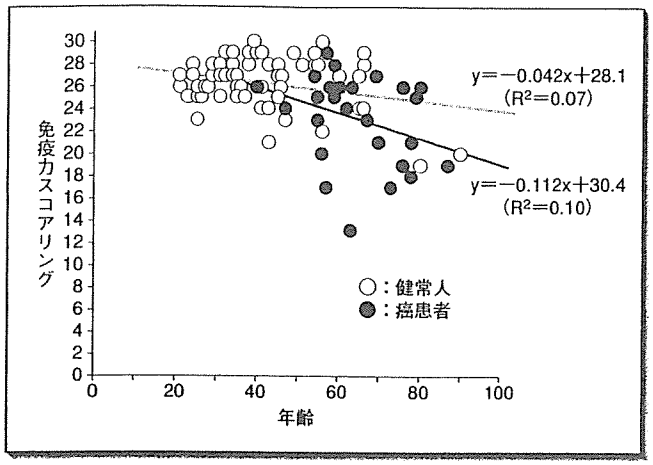

図2．免疫力スコアリングの加齢変化
10項目の免疫細胞マーカーと機能について測定し，3段階のスコアを与え，その総和を免疫力スコアリングとした。その分布は健常人では20～30の間に分布し，加齢とともに緩やかに低下する。一方癌患者では13～29の間に分布し，健常人とは異なった分布状態を示し，加齢に伴う変化も急である。

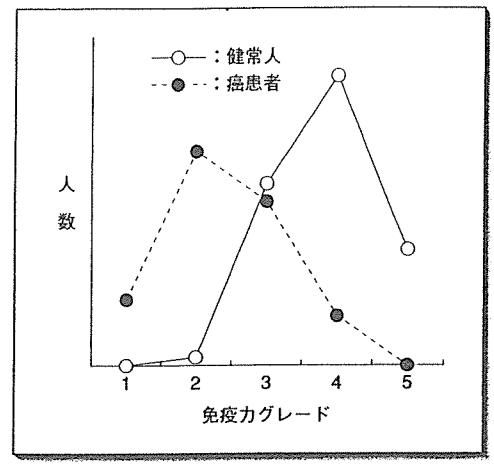

図3．免疫力の加齢変化と疾病による低下の促進
免疫力スコアリングをさらに5段階に分けると，健常者の免疫力グレードは3～5に分布するが，癌患者では2にピークがあり，2～4に分布する。一見して，癌患者の免疫力グレードの低いことがわかる。

2．組織または細胞の移植

①骨髄移植，②免疫細胞移植，③胸腺移植（動物モデルのみ）

いずれも，文献において詳述[1)2)4)5)]しているので，ここでは代表的なもの3つについて簡略な説明にとどめる。

1）栄養調節

免疫機能回復には栄養調節が第一の選択である。一言でいえば，バランスと調和のある食事が重要である。栄養失調でも，栄養過剰でも免疫機能は下がる。齧歯類におけるカロリー制限による寿命延長がオーバーに宣伝されているが，日本人の戦後の平均寿命の延長の一因は栄養改善であったことを忘れてはならない。

2）抗酸化物質

生き物の多くは酸素呼吸によりエネルギーを得ているが，その際発生する活性酸素に必ず曝される。無論その活性酸素を無毒化する抗酸化システムが生体には備わっている。その抗酸化システムは適度な運動により，その機能が上昇することが知られている。しかし，運動が過度になれば，抗酸化システムで処理しきれない活性酸素が増加する。年余にわたってそうした活性酸素に曝されることが，生体の老化の一因であるといわれている。

自然界には抗酸化機能をもつ物質がたくさんある。サプリメントとして市場に出ている多くのものは，その抗酸化機能を強調しているが，それらの免疫機能回復効果については，まだ十分なエビデンスが少ないのが現状である。問題は，抗酸化物質による免疫機能回復効果が個々人の遺伝的背景により異なることである。

3）活性化T細胞輸注

個体の末梢血液から採取したリンパ球を分離し，特殊条件下で培養すると500～1,000倍に増殖させることができる。この方法は一般的には癌に対する免疫療法として利用されているが，非特異的に増殖したリンパ球に癌細胞傷害効果を期待することは難しい。しかし，非特異的に増殖させた活性化T細胞は免疫機能を十分回復することができる。免疫機能の低下のパターンは人により異なるため，それぞれに合わせて，リンパ球を調整するオーダーメイド輸注も可能である。培養するリンパ球は病気になってから採血するより，若く元気のよいときのものを使ったほうがよいので，それには，リンパ球を保存するT細胞バンクのようなものが必要になる。

おわりに

免疫系が我々の身体にとって重要な役割を果たしており，その機能が老化やさまざまな環境因子により変化する

ことは周知のこととなっている。しかし，自分の免疫系の機能が，どの程度なのか知っている人は少ない。それは，個体の免疫機能のレベルを総合的に評価する方法がなかったからである。

本稿では，多様な細胞からなり，さまざまな機能を示す免疫系の機能を包括的に測定し，総合的な免疫力のレベルとして評価する方法を提示した。その免疫力に低下があるなら，免疫力を回復させることが健康維持には最も重要である。ヒトの遺伝的背景は極めて多様である。その多様性に富んだ万人の誰にも有効な回復方法はない。実際には，ある免疫回復方法がどの程度効果があるのか，免疫力を評価・モニターしながら，自分に合う方法をみつける必要がある。免疫力の回復をうたっている多くのサプリメントについても，摂取後の免疫力の評価を行い，科学的なエビデンスを用意することが望まれる。以上，免疫機能の評価方法と回復方法の重要性について概説した。

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INDUCTION OF IMMUNOLOGICAL TOLERANCE BY ORAL, BUT NOT INTRAVENOUS AND INTRAPORTAL, ADMINISTRATION OF OVALBUMIN AND THE DIFFERENCE BETWEEN YOUNG AND OLD MICE

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Abstract: Background: Induction of immunological tolerance is dependent on the route of antigenic administration, the dose of an antigen and the age of animals. Objectives: We investigated the effect of age on the tolerance induction in mice by administration of antigen through different routes and at different doses. Design: Young and old BDF1 mice were orally, intraportally or intravenously administrated with a low or a high dose of ovalbumin (OVA). Then, delayed-type hypersensitivity (DTH) responses and serum anti-OVA antibody levels were assessed after systemic immunization of OVA with alum after appropriate intervals. Results: In the young mice, oral administration of OVA suppressed DTH response and anti-OVA IgG1, IgG2b, IgM and IgE level in a dose-dependent manner. In the old mice, however, the suppression of IgG1 and IgE levels was induced by oral administration of a low dose of OVA, but no suppression by a high dose. On the other hand, intraportal or intravenous injection of OVA did not suppress DTH response and enhanced anti-OVA antibody levels in a dose-dependent manner in both young and old mice. Production of anti-OVA IgG2a antibody after systemic injection of OVA was detected in the mice, which had been treated with intraportal or intravenous injection of OVA, but not detected in the mice, which had been treated with oral administration of OVA. On the contrary, suppression of anti-OVA IgE antibody was observed only in the mice, which had been treated with oral administration of OVA. Conclusion: The oral administration of OVA, neither intravenous nor intraportal, induced immunological tolerance to OVA. An adequate dose of OVA for the tolerance induction and the suppression of antibody production are different between young and old mice. The suppression of IgE antibody was observed only by oral administration of OVA, much obviously in young mice than in the old. The results also indicated that the antigen processing in the liver did not play a major role in the induction of oral tolerance to OVA.

Key words: Immunological tolerance, ovalbumin, oral administration, intraportal administration, intravenous administration, aging.

Introduction

Food is essential to support our body, but contains a lot of antigenic materials. However, our body usually does not respond to antigenic materials in the food. In other words, when an antigen is given per os, antibody response is suppressed after systemic administration of the same antigen. This unresponsiveness induced by oral administration of antigen is called oral tolerance (1,2). The unresponsiveness to antigen or tolerance can be also induced by administration of a large dose of antigen by systemic route, either intravenously or intraperitoneally. Thus, tolerance is an important mechanism to prevent adverse immune response to self-antigens and food materials.

Oral tolerance is easily induced in young adult animals, but hard in neonatal and old mice (3,4). In a previous paper (5), we reported that oral administration of an antigen (sheep red blood cells: SRBC) induced tolerance to SRBC in young mice, but enhanced the antibody response to SRBC in old mice.

An antigen given orally is first absorbed by intestinal mucosa and flows into the systemic circulation through the liver. In other words, the antigen is first processed in the intestinal mucosa and then in the liver. Thus, the difference in the induction of oral tolerance between young and old mice could be ascribed to age-related changes in immune function and antigen processing in the digestive tract and liver.

It is known that both multiple feeding of a low dose of an antigen, and single or multiple feeding of a high dose of an antigen induces oral tolerance. It was shown that mechanisms of oral tolerance were different depending on the dose of an antigen. Multiple feedings of a low dose (1 mg) of an antigen generated cells producing inhibitory cytokines, whereas a high dose (20 mg) of an antigen induced clonal anergy (6).

There are other routes by which administration of an antigen induces tolerance. It was shown that intraperitoneal (i.p.) (7,8), intraportal (9,10,11) and intravenous (i.v.) (12) injection of an antigen could induce tolerance. Inhibition of collagen-induced arthritis could be seen by oral administration (13), intraportal injection (11) or i.v. injection (14) of type Ö† collagen. With ovalbumin (OVA), it was reported that tolerance was induced

by oral administration (1) or i.v. injection (15) of OVA and i.p. or subcutaneous (s.c.) injection of OVA/IFA (16).

In a previous paper (5), we employed SRBC as an antigen and found an age difference in the induction of the immunological tolerance. In the present study, we employed OVA as an antigen and examined the influence of various dose of OVA and age of host on the induction of oral tolerance. As compared with SRBC, we could obtain more precise immunological data by using OVA, including delayed-type hypersensitivity (DTH) and various isotypes of anti-OVA antibodies. In addition, we compared the tolerance induction by different routes of OVA-administration; oral, intraportal and intravenous. The findings suggested that the role of the liver was marginal in the induction of oral tolerance to OVA and discussion was made on the different response between young and old mice.

Materials and methods

Mice

BDF_1 male mice were used in the present study. Young mice (3 months of age) were purchased from SLC Japan (Shizuoka, Japan). They were housed in a specific pathogen-free colony at the Tokyo Metropolitan Institute of Gerontology. Old mice (19 months of age) were obtained from the aging colony of Tokyo Metropolitan Institute of Gerontology. The mean life span of BDF1 male mice reared in the colony was 954 ± 215 days.

Oral administration of OVA

Ovalbumin, Chicken egg (OVA, grade V, Sigma, St. Louis, MO) was used as an antigen. Mice were orally administered 25mg or 0.25 mg of OVA in 1 ml of saline daily for 5 successive days as shown in Fig. 1A. As a control, the same volume of saline was given in the same way.

Intraportal Injection of OVA

Anaesthetized mice underwent a laparotomy and filtered 25mg or 0.25mg of OVA in 300 ml of 0.03% trypan blue-saline was injected into the portal vein as shown in Fig. 1B. As controls, 0.03% trypan-blue saline was injected in the same way. In this case, OVA solution was colored by adding the trypan blue to confirm that it was really injected into the liver through the portal vein. After injection, bleeding from the portal vein was stopped with thrombinÅiMochida, Tokyo, JapanÅjand then the peritoneum and the skin were sutured.

I.v. injection of OVA

Mice were anaesthetized and injected with filtered 25mg or 0.25mg of OVA in 250 ml of saline into the tail vein as shown in Fig. 1C. As controls, saline was injected in the same way.

Immunization with OVA and alum

One week after the first feeding or injection of OVA into tail vein or portal vein, mice were injected intraperitoneally with 50 mg of OVA and 4 mg of Al(OH)3 in 0.5 ml of saline. The second immunization was performed 2 weeks after the first one in the same manner. The third immunization (i.p. injection of 0.5 mg of OVA in 0.5 ml of saline) was performed about 8 weeks after the second one.

Delayed-type hypersensitivity (DTH) response

Seven weeks after the second immunization, 20 mg of OVA in 20 ml of saline was intradermally injected into the right ear. As a negative control, 20 μl of saline was injected into the left ear. The thickness of the ear was measured using a dial thickness gauge (Ozaki MFG. CO., LTD. Tokyo, Japan) before and 24 hours after the challenge injection. The swelling rate of the ear was calculated as follows: (thickness of the ear 24 hr after the challenge - thickness of the ear before the challenge)/ thickness of the ear before the challenge X 100 %

Assessment of anti-OVA antibody by enzyme-linked immunosorbent assay (ELISA)

The serum levels of OVA-specific antibodies were analyzed by the modified method as previously described (17). Briefly, 96-well flat-bottomed microtiter plates (Immulon 1, Dentate Laboratories, Inc., Virginia, USA) were coated with optimal concentrations of OVA (100 μl/well) dissolved in carbonate buffer (pH 9.6) at 4°C overnight. OVA of 10 μg/ml was coated for the assay of anti-OVA IgG1, IgG2a, IgG2b and IgM, and 50 μg/ml of it was coated for the assay of IgE. Then, the wells were washed three times with PBS containing 0.2 % gelatin and 0.05 % Tween 20. After washing, 50 μl of each serum sample diluted appropriately in PBS was added to the wells and they were incubated at room temperature (RT) for 2 hours. After washing, the wells were added with biotinylated anti-mouse IgG1, IgG2a, IgG2b, IgM or IgE (PharMingen, San Diego, CA) and incubated at RT for 30 minutes (min). After washing, The wells were reacted with horseradish peroxidase linked streptavidin (DAKO A/S, Glostrup, Denmark) at RT for 30 min. After washing, the enzyme reaction was performed with o-phenylene diamine (Sigma, USA) in sodium citrate buffer (pH 5.4) in the presence of H_2O_2 for 15 min in the dark. It was interrupted by the addition of 25 μl of 2 N HCl, and the absorbance was measured at 490 nm.

Statistical Analysis

The significance of the difference (e.g., p values) between groups was evaluated by the Mann Whitney U test using a Statview program designed for Macintosh computers.

Results

Suppression of DTH response by oral administration of OVA in young mice, but not by either intrapotal or i.v. injection of OVA

We first investigated the influence of the age of mice, the dose of OVA and the route of administration on the tolerance induction in DTH response. Therefore, young and old BDF1 mice were orally, intraportally or intravenously administrated

Figure 1

Experimental designs. A: Experimental design for induction of oral tolerance and assessment for immune responses. Young and old BDF1 mice were orally administrated with 25 mg or 0.25 mg of OVA or saline, and then immunized intraperitoneally (i.p.) with OVA plus alum twice and with OVA once. Serum samples were collected for assessment of anti-OVA antibody levels, and DTH response was assessed. B: Experimental design for intraportal injection of OVA and assessment for immune responses. Young and old BDF1 mice were injected with 25 mg or 0.25 mg of OVA into the portal vein, and then immunized i.p. with OVA and alum twice. Serum samples were collected and DTH response was assessed. C: Experimental design for intravenous injection of OVA and assessment for immune responses. Young and old mice were intravenously injected with OVA or saline. Immunization and assessment of immune responses were performed in the same manner as that in B.

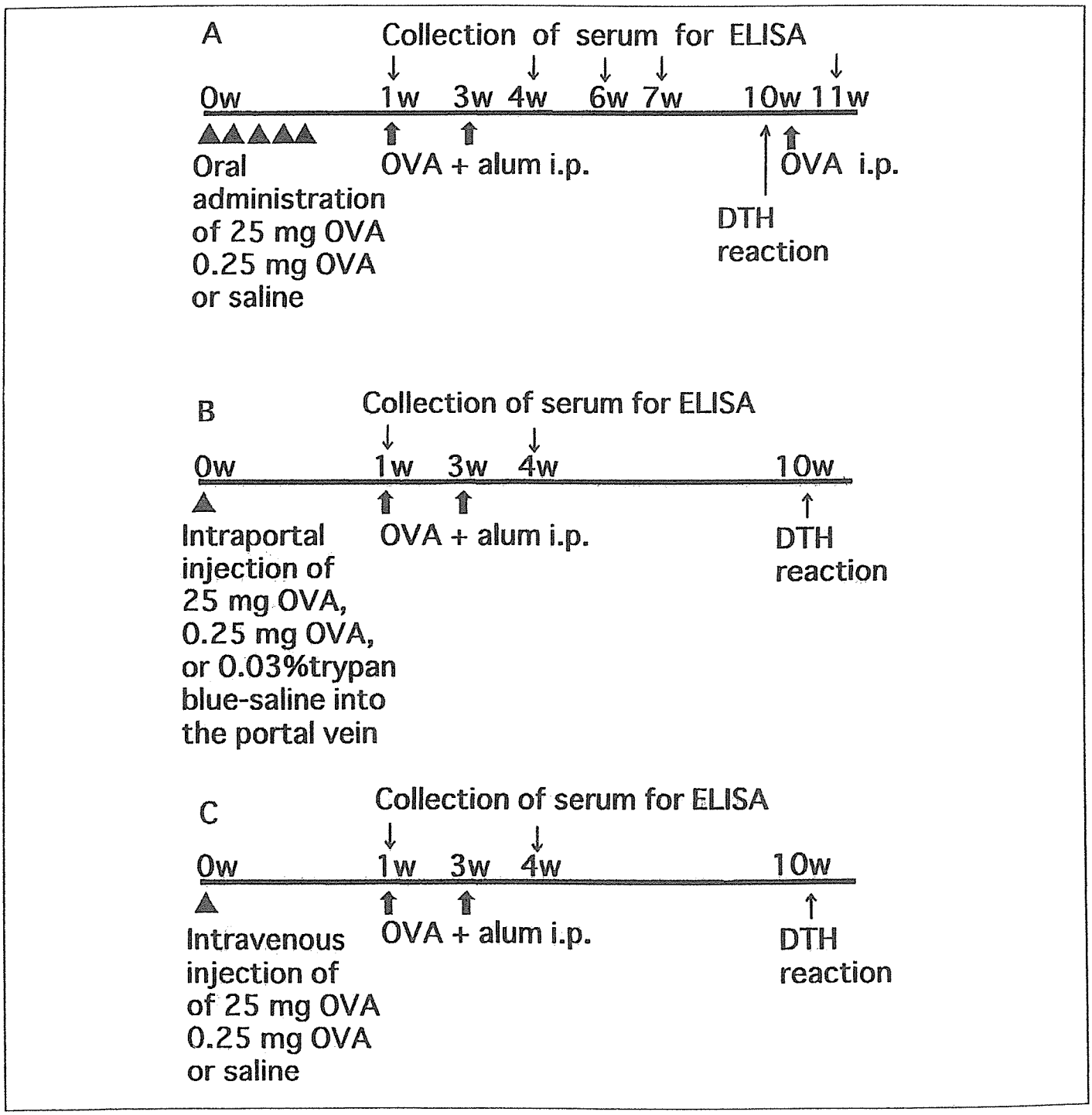


Table 1
DTH responses to OVA in young and old mice previously treated with OVA #

Route of administration	Mice	Administered with	N=	Rate of swelling (%) Injected with 20μg of OVA	Rate of swelling (%) Injected with saline
A. Oral	Young	Saline	8	74.5 ± 7.5	4.1 ± 2.7
		0.25mg OVA	8	29.7 ± 7.5 *	6.1 ± 3.2
		25mg OVA	7	8.7 ± 4.1 *	8.0 ± 3.9
	Old	Saline	6	28.2 ± 10.0	15.0 ± 3.1
		0.25mg OVA	5	26.6 ± 6.8	18.2 ± 4.9
		25mg OVA	6	28.9 ± 8.8	13.5 ± 4.8
B. Intraportal	Young	Saline	6	59.5 ± 10.8	9.4 ± 3.9
		0.25mg OVA	8	48.5 ± 7.2	7.7 ± 3.3
		25mg OVA	8	42.6 ± 10.0	4.6 ± 2.6
	Old	Saline	3	13.4 ± 3.2	8.4 ± 4.2
		0.25mg OVA	1	55.6	10.4
		25mg OVA	2	32.8 ± 4.9	4.9 ± 6.2
C. Intravenous	Young	Saline	4	87.1 ± 23.7	13.5 ± 8.9
		0.25mg OVA	4	123.1 ± 22.7	8.3 ± 6.7
		25mg OVA	4	98.1 ± 12.1	3.8 ± 3.0
	Old	Saline	4	84.3 ± 9.4	10.3 ± 1.7
		0.25mg OVA	4	71.8 ± 14.6	3.7 ± 4.1
		25mg OVA	3	100.1 ± 32.2	10.3 ± 10.3

DTH was assessed about 10 weeks after the first administration of OVA. Mice were injected with 20 mg of OVA into the right ear intradermally. As negative control, saline was injected into the left ear. The thickness of the ear was measured before and 24 hours after the challenge injection. The results are displayed as the mean ± standard error of the mean (SEM). Statistical significance is shown as *($p<0.005$), compared with the value of age-matched mice administered with saline by the same route.

with a high or low dose of OVA, and DTH responses were assessed before and after systemic immunization with OVA with adjuvant. Experimental protocols were shown in Fig. 1. DTH responses to OVA were assessed about 7 weeks after the second systemic immunization (10 weeks after the first oral administration, intraportal injection or i.v. injection of OVA). The results obtained were summarized in Table 1. The rate of ear-swelling was suppressed in young mice orally administrated with OVA ($p<0.005$), compared with that of mice orally administered with saline (control group) in Table 1A. The magnitude of suppression was higher in the mice given a high dose than those given a low dose of OVA (Table 1A). On the other hand, no difference was observed among the three groups of old mice as shown in Table 1A.

The experimental design of intraportal injection of OVA and the result of DTH response were summarized in Fig. 1B and Table 1B, respectively. In young mice, the previous injection of OVA into the portal vein did not influence DTH response and the swelling rates were almost the same among the three groups (Table 1B). Whereas, a slight enhancement of swelling rate was observed in old mice injected with a high dose of OVA into the portal vein as shown in Table 1B.

The experimental design of i.v. injection of OVA and the results of DTH response were shown in Fig. 1C and Table 1C. The i.v. injection of OVA also did not influence DTH response and no significant difference was seen among the three groups of both young and old mice (Table 1C).

In these experiments, no significant swelling was observed when saline was injected into the ear (Table 1).

Dose-dependent suppression of serum anti-OVA antibodies in young mice by feeding of OVA, and suppression in old mice by feeding of low dose of OVA

Serum samples were collected after the oral administration of OVA, and analyzed for anti-OVA IgG1, IgG2a, IgG2b, IgM and IgE levels. The results obtained were summarized in Fig. 2. In young mice orally administrated with a high dose of OVA, a significant suppression of serum anti-OVA IgG1 level was observed 4 weeks after the first feeding. The magnitude of suppression of IgG1 production was dependent on the dose of OVA. The suppression by a high dose feeding of OVA continued for at least 11 weeks after the first oral administration.

In old mice, however, only the low dose feeding of OVA significantly suppressed anti-OVA IgG1 level, while the high dose feeding did not influence IgG1 levels (Fig. 2).

In young mice, a high dose feeding of OVA significantly suppressed anti-OVA IgG2b levels after the third immunization (11 weeks after the first feeding). A significant suppression of serum anti-OVA IgM levels of young mice ($p< 0.005$) was also observed in both the high and the low dose feeding group after 11 weeks. In case of anti-OVA IgE antibody response of young

Figure 2

Dose-dependent suppression of serum anti-OVA antibodies in young mice by oral administration of OVA and suppression of those by a low dose feeding in old mice. Serum samples after oral administration of OVA were assessed for anti-OVA IgG1, IgG2a, IgG2b, IgM and IgE levels. One week after oral administration corresponds the day of the first immunization of OVA and alum. Two weeks after oral administration corresponds 1 week after the first immunization. Four, 6 and 7 weeks after oral administration correspond 1, 3 and 4 weeks after the second immunization. Eleven weeks after oral administration corresponds 1 week after the third immunization. Serum samples were diluted 500 folds for IgG1 assay, 10 folds for IgG2a, IgG2b and IgM assay, and 2 folds for IgE assay. The ordinate shows OD (490nm). The results are displayed as the mean + SEM. Open, dotted and closed columns represent the levels in mice orally administered with saline, 0.25 mg of OVA and 25 mg of OVA, respectively. The numbers of young mice orally administered with saline, a low dose of OVA, or a high dose of OVA were 8, 8, or 6-8, respectively. The numbers of old mice orally administered with saline, a low dose of OVA, or a high dose of OVA were 5-6, 5-7, or 6-7, respectively. Statistical significance is shown as *($p<0.05$) and **($p<0.005$), compared with the value of age-matched mice orally administered with saline.

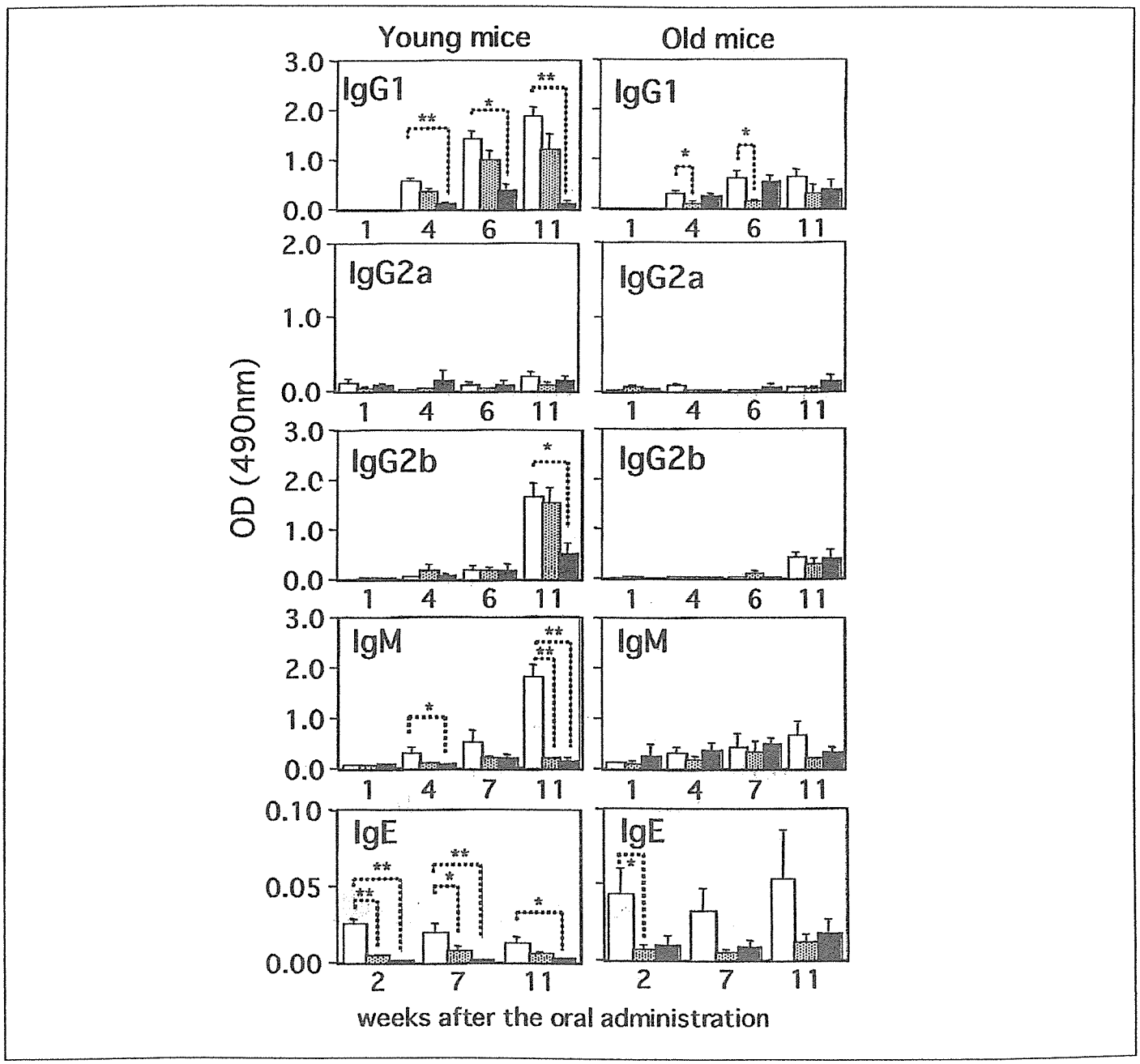
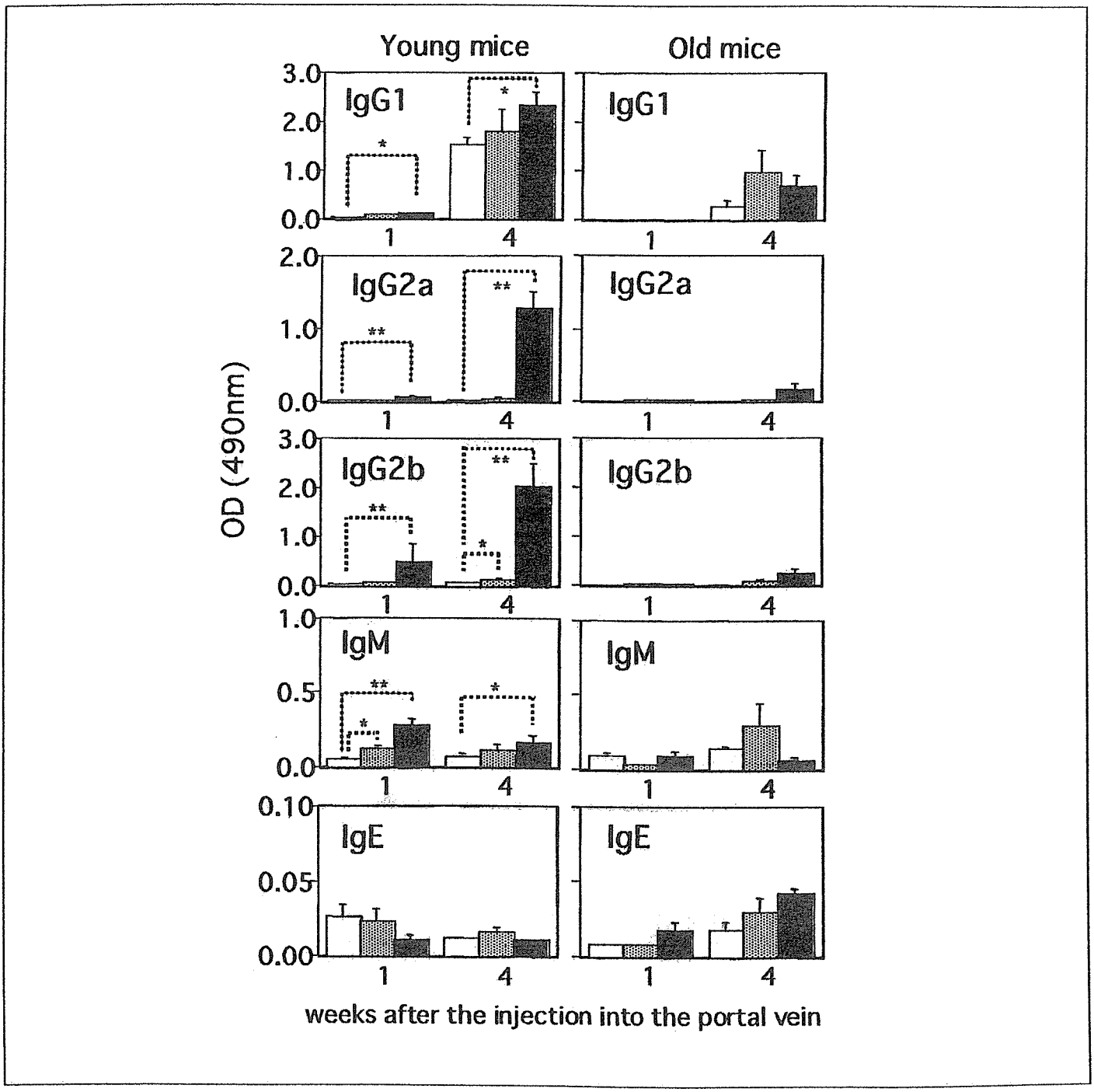


Figure 3

Enhancement of serum anti-OVA antibody in young and old mice by intraportal injection of OVA. Serum samples after intraportal injection of OVA were assessed for anti-OVA IgG1, IgG2a, IgG2b, IgM and IgE levels. One week after the intraportal injection of OVA is before the first immunization of OVA and alum. Four weeks after the injection mean 1 week after the second immunization. Serum samples were diluted 500 folds for IgG1 assay, 10 folds for IgG2a, IgG2b and IgM assay, and 2 folds for IgE assay. The ordinate shows OD (490nm). The results are displayed as the mean + SEM. Open, dotted and closed columns represent the levels in mice injected with saline, 0.25 mg of OVA and 25 mg of OVA, respectively. The numbers of young mice intraportally injected with saline, a low dose of OVA, or a high dose of OVA were 6, 8, or 8, respectively. The numbers of old mice intraportally injected with saline, a low dose of OVA, or a high dose of OVA were 3, 2, or 2, respectively. Statistical significance is shown as *($p<0.05$) and **($p<0.005$), compared with the value of age-matched mice injected with saline into the portal vein.



mice, a significant suppression was detected 2 weeks after the first feeding, and the suppression by a high dose feeding was observed during the experimental period.

In old mice, however, only the low dose feeding of OVA significantly suppressed anti-OVA IgE level at 2 weeks, while no significant response and difference were observed in the serum levels of anti-OVA IgG2b and IgM as far as examined (Fig. 2).

Serum anti-OVA IgG2a levels were never enhanced in young and old mice which had been treated with oral administration of OVA, followed by systemic the systemic immunization.

Dose-dependent enhancement of serum anti-OVA antibodies in the young and old mice injected with OVA into the portal vein

Serum samples after the injection of OVA into the portal vein were collected and assessed for anti-OVA Ig levels. The results obtained are summarized in Fig. 3. In young mice injected with OVA into the portal vein, a significant enhancement of serum anti-OVA IgG1 level was observed in a dose dependent manner 1 and 4 weeks after the injection. In old mice, intraportal injection of OVA enhanced IgG1 levels 4 weeks after the injection, although a significant difference was not observed.

A significant enhancement of serum anti-OVA IgG2a and IgG2b levels was observed 1 week and 4 weeks after the intraportal injection of a high dose of OVA in young mice. A slight enhancement of IgG2a and IgG2b levels was observed in old mice 4 weeks after the injection of the high dose, but no significant effect was observed in IgG2a and IgG2b levels of old mice.

A significant and dose-dependent enhancement of anti-OVA IgM levels was observed in young mice 1 week after the injection of OVA (before the systemic immunization). In old mice, however, no significant enhancement of serum anti-OVA

Table 2

Enhancement of serum anti-OVA antibody in young and old mice by intravenous injection of OVA #

Mice			Young	
I.v. injected with		saline	0.25mg OVA	25mg OVA
N=		4	4	4
IgG1	1w	0.004 ± 0.004	0.010 ± 0.006	0.204 ± 0.052 *
	4w	1.403 ± 0.043	1.806 ± 0.138 *	1.806 ± 0.154 *
IgG2a	1w	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
	4w	0.065 ± 0.059	0.039 ± 0.011	1.431 ± 0.059 *
IgG2b	1w	0.000 ± 0.000	0.000 ± 0.000	0.500 ± 0.303 *
	4w	0.372 ± 0.334	0.527 ± 0.099	1.516 ± 0.052 *
IgM	1w	0.000 ± 0.000	0.000 ± 0.000	0.181 ± 0.072 *
	4w	0.012 ± 0.007	0.051 ± 0.022	0.535 ± 0.246 *
IgE	1w	0.0039 ± 0.0004	0.0054 ± 0.0004 *	0.0044 ± 0.0004
	4w	0.0068 ± 0.0018	0.0126 ± 0.0058	0.0050 ± 0.0004
Mice			**Old**	
I.v. injected with		**saline**	**0.25mg OVA**	**25mg OVA**
N=		**4**	**4**	**3**
IgG1	1w	0.000 ± 0.000	0.114 ± 0.010	0.069 ± 0.022 *
	4w	1.526 ± 0.010	1.890 ± 0.196	1.719 ± 0.205
IgG2a	1w	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
	4w	0.002 ± 0.002	0.003 ± 0.002	0.417 ± 0.092 *
IgG2b	1w	0.000 ± 0.000	0.001 ± 0.001	0.011 ± 0.006
	4w	0.037 ± 0.018	0.188 ± 0.105	1.280 ± 0.223 *
IgM	1w	0.040 ± 0.025	0.053 ± 0.022	0.109 ± 0.034
	4w	0.107 ± 0.024	0.110 ± 0.048	0.255 ± 0.155
IgE	1w	0.0040 ± 0.0004	0.0061 ± 0.0013	0.0050 ± 0.0003
	4w	0.0110 ± 0.0028	0.0156 ± 0.0023	0.0088 ± 0.0016

Serum samples after the intravenous injection of OVA were assessed for anti-OVA antibody levels. One week after the injection of OVA corresponds the day of the first immunization of OVA and alum. Four weeks after the injection corresponds 1 week after the second immunization. Serum samples were diluted 500 folds for IgG1 assay, 10 folds for IgG2a, IgG2b and IgM assay, and 2 folds for IgE assay. The results are displayed as the mean OD ± SEM. Statistical significance is shown as *($p<0.05$), compared with the value of age-matched mice intravenously injected with saline.

IgM levels was detected during the experimental period.

Regarding anti-OVA IgE, the intraportal injection of OVA did not enhanced the levels in young mice, whereas in old mice injected with a high dose of OVA into the portal vein, a slight enhancement of anti-OVA IgE level was observed at 4 weeks (Fig. 3).

Dose-dependent enhancement of serum anti-OVA antibodies in the young and old mice intravenously injected with OVA

Serum samples after the i.v. injection of OVA were collected and assessed for anti-OVA Ig levels in the same way as intraportal injection. The results obtained are summarized in Table 2, and they were almost the same as those of mice injected with OVA into the portal vein. In young mice intravenously injected with OVA, a significant enhancement of serum anti-OVA IgG1 level was observed at 1 and 4 weeks after the injection in a dose dependent manner. In old mice, serum anti-OVA IgG1 level was also enhanced by the i.v. injection of OVA at 1 week.

A significant enhancement of serum anti-OVA IgG2a and IgG2b levels was observed 4 weeks after the i.v. injection of a high dose of OVA in both young and old mice.

A significant enhancement of anti-OVA IgM levels was seen in young mice 1 week and 4 weeks after the i.v. injection of OVA. However, in old mice, a significant enhancement of serum anti-OVA IgM levels was not detected during the experimental period.

Regarding anti-OVA IgE level, a significant difference was not observed in young and old mice intravenously injected with OVA (Table 2).

Discussion

We have previously shown that oral administration of SRBC to young mice induced oral tolerance, but the same treatment induced the enhancement of anti-SRBC antibody response in old mice (5). The present experiment was designed to see whether the similar age effect can be seen by oral administration of ovalbumin. The results showed that the enhancement of antibody response to OVA was not observed in old mice as observed in that to SRBC. On the contrary, suppression of antibody response was observed by oral administration of a low dose of OVA, but not by a high dose. In DTH response, tolerance induction to OVA was observed in young mice but not in the old. The resistance to the tolerance induction observed in the present paper was consistent with a report by Faria et. al. (4) that mice at 8 weeks of age were susceptible to the induction of oral tolerance to OVA but those at 24 weeks of age became refractory. Friedman et. al. (6) reported that feeding of a low dose of an antigen was shown to generate cells producing inhibitory cytokines, whereas high doses of the antigen induced clonal anergy. According to this idea, old mice become resistant to the induction of clonal anergy by oral administration of antigen, but still possible to produce inhibitory cytokines. According to Koga et. al. (18), synthesis of both Th1 and Th2-type cytokines by the splenic CD4+ T cells was significantly diminished in aged mice orally immunized with OVA plus cholera toxin, compared with young mice treated in the same manner. However, production of inhibitory cytokines may be not markedly diminished in aged mice.

The second point of interest in the present study was that tolerance was induced by oral administration, but not by intraportal or intravenous injection of OVA. Other antigens such as allogeneic cells (9), Schistosome eggs (10) and type II collagen (11) are known to induce tolerance by intraportal administration. Regarding allogeneneic cells, their trapping by the hepatic component appeared to be central to the induction of tolerance (19). Antigen presentation by Kupffer cells and lymphocyte proliferation in the liver are necessary for the induction of oral tolerance by intraportal administration of an antigen (20). Furthermore, Viney et. al. (21) reported that mice treated with Flt3L (a growth factor that expands DC in vivo) showed enhancement of the induction of oral tolerance. On the other hand, Limmer et al. (22) reported that liver sinusoidal endothelial cells (LSEC) could play a role in the induction of antigen specific T cell tolerance. These reports favor that the liver is playing a role in the induction of tolerance to exogenous antigen.

In the present paper, however, the injection of OVA into portal veins could not induce tolerance but enhanced immune response to OVA. The results suggest the intestinal mucosa appears to be more important than the liver in the processing of antigen for the oral tolerance and is consistent with the findings by Bruce and Ferguson (23). They reported that tolerance was observed in mice injected with serum from OVA-fed mice, but not in those injected with serum containing systemically filtered OVA. The gut mucosa of old animals appears to lose the capacity to process food, and can process a limited amount of OVA. A part of OVA which is not processed by the gut may flow into the systemic circulation and act as antigenic material in old mice. The present study clearly shows that OVA injected into blood circulation can not induce tolerance, but enhance anti-OVA immune responses.

Karlsson et. al. (24) reported that the tolerance could be mediated by CD25-positive cells. If that is the case, OVA processed in the gut mucosa can activate CD25 positive cells.

In the present study, the suppression of IgE antibody is clearly seen by oral administration of OVA, significantly in young mice and slightly in the old. The data suggest that administration of an antigen by oral, rather than by systemic route, may be effective in suppressing possible allergic state to the antigen.

In conclusion, immunological response to OVA was examined in young and old mice, using three different routes of administration and two different dose of the antigen. The results showed the oral administration of OVA, neither

intravenous nor intraportal, induced immunological tolerance to OVA. An adequate dose of OVA for the tolerance induction and the suppression of antibody production are different between young and old mice. The suppression of IgE antibody was observed only by oral administration of OVA, much obviously in young mice than in the old. The results also indicated that the antigen processing in the liver did not play a major role in the induction of oral tolerance to OVA.

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PROPER ASSESSMENT AND RESTORATION OF IMMUNOLOGICAL FUNCTION FOR THE IMPROVEMENT OF QOL AND ELONGATION OF HEALTHY LIFESPAN IN THE ELDERLY.

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Abstract

The lifespan of Japanese people ranks top in the world, but the healthy lifespan is 5 or 6 years less. The fact has indicated that many elderly people are sick or bed ridden for several years before their death. The issue is how to improve QOL for the last several years before death and to extend healthy lifespan.

One approach to this purpose is reconstruction or restoration of the impaired immune functions of the elderly people. For this, we need the proper assessment of whole immunological functions of the elderly people. We are employing comprehensive assessment of various immunological indices mainly of T cells which is called SIV.

Most of methods for immunological restoration reported are still at the level of animal models. Broadly speaking, there are two ways; one is activation of existing immune cells in individuals, and the other is transplantation of active tissues or immune cells from young donors.

The former includes caloric restriction, anti-oxidants, hormones and various supplements. The latter is grafting of tissues and cells. At the present time, for human application, the infusion of autologous activated T cells prepared from peripheral blood lymphocytes of each individual would be one of the most effective methods to restore the immunological state.

Peripheral T cells can be expanded 100 – 1000 fold in vitro culture for 2 weeks with recombinant IL-2 and immobilized monoclonal anti-CD3 antibody. Ten billion (10^{10}) of T cells prepared from 10 - 20 ml of peripheral blood can be returned to individual for immunological restoration. When necessary, specific subpopulation of T cells can be removed before the infusion.

The best source of T cells for in vitro expansion is those from young adults. For this purpose, we need T cell bank system where one can, when young, can store one's own lymphocytes in liquid nitrogen for many years. When necessary at the time of illness or senescence, one can ask the T cell bank to propagate one's own lymphocytes in vitro and receive the infusion of autologous activated T cells.

The effectiveness of this method was already confirmed in children with congenital immune deficiency, and in a mouse model using C57BL/6.Thy-1.2 and B10.Thy-1.1.

1. Infection is a major cause of death in the elderly.

In most developed countries, the three leading causes of death are cancer, brain stroke and cardiac infarction, and infections such pneumonia rank 4th or 5th (1). This information is based upon death notices presented to the MHW from physicians throughout the country. These are written based on the clinical and laboratory findings, but without autopsy findings in most cases.

Actually in Japan, approximately 1 million people die of various diseases per year and less than 3 % are transferred for autopsy examination. Autopsy revealed that causes of death varied by hospital, but infections were one of the major causes (1).

There are two reasons why infection is one of the major causes or the top cause of death in most hospitals. One is an increase of elderly patients with immunologically deficient state, and that many cancer patients die of infection. In our survey, infectious

lesions were found in about one third to half of cancer patients and half of them die of infections such as pneumonia or urinary tract infection. In other words, about one sixth to a quarter of cancer patients die of infection and this promotes infection to the major cause of death by autopsy examination in Japan and probably in most other countries.

I am now working as a pathologist in a hospital with 300 beds in Tokyo. Over the last 7 years, 140 autopsies have been carried out. When looking at clinical diagnosis, the top disease is cancer and the second is infection, but when analyzing cancer cases from a pathological viewpoint, about half are suffering from infections and a quarter die of infections as mentioned above. Therefore, among 140 autopsy cases, the top cause of death was infection, reaching about 40 % of total cases.

That infection is the leading cause of death in autopsy cases is also reported by other hospital. For instance, Mac Gee (2) in Geneva examined 3000 autopsy cases and reported that the top cause of death in the elderly was infections such as bronchopneumonia and urinary tract infection, exceeding 50 % of the total (Table-1). There are now more than 25,000 centenarians in Japan. Although small in number, some cases were examined by autopsy and about 2/3 of them died of infections such as pneumonia and sepsis (3).

Most cancer patients, nowadays, are treated with operation, anti-cancer drugs and radiation, and a considerable number of cases are successfully treated and survive. But it is of importance that many cancer patients become susceptible to infection, because immune functions are declining due to old age and cancer treatments are generally immuosuppressive.

In practice, the immune deficient state is caused by variable factors such as aging, stress, genetic background, inappropriate life style, disease per se and various therapies for disease, especially for cancer. Thus, immunological restoration is the most suitable solution in this case. Although it is obvious that many patients die of infections, precise immunological data are not available in many cases.

Japanese clinical doctors of course know the importance of the immune system against infection, but they do not assess immune function. There are two reasons for this: The first is that they do not know what kind of immunological functions to assess and the second is that the assessment cost is generally not covered by insurance. So at present time, we need the guide line what kind of immune functions should be assessed and this is quite necessary for immunological restoration.

Another important point is that the immune system is not only acting as the defense system against infection, but also playing major role in the maintenance of the internal environment together with nervous and endocrine systems. In another words, a network composed of nervous, endocrine and immune system is an organization against stress in a broad sense. Therefore, immune deficient state gives rise to the susceptibility to infection as well as to deterioration of QOL. In any event, the immune system is combating against infection and stress. So when depressed, it should be reinforced by any means. For this purpose, we need proper assessment of immunological state.

2. Assessment of the immunological state

The immunological state is important information for patients suffering from various diseases and frail elderly people (4). In many cases, however, immunological indices regarding immunological state are not assessed, because it is unknown what kind of immunological indices are suitable for the assessment of the whole immunological

vigor of individuals. This is mainly due to polymorphism of immunological state or the immune system. When talking about the immune system, most people think of the acquired immunity of lymphocytes. Acquired immunity per se is a complicated organization, composed of various cells, tissues and factors produced by these immune cells. It is difficult to say which cells, tissue or factors are most reflective of the whole immune vigor of individual people. We have finally arrived at an interim conclusion that the comprehensive analysis of several immunological indices and functions is reasonable for the assessment of whole immunological vigor and called it the scoring of immunological vigor (SIV).

We have reported that T cells functions are most vulnerable to aging (1) and this is mainly caused by thymic involution starting in early puberty. Age-related changes of the T cell dependent immune system are observed in decreases T cell number, a change in T cell subsets and in qualitative changes such as proliferation and cytokine production. Therefore, T cells and their characteristics should be core indices for the immunological state and eventually we selected 10 indices as follows: 1) number of T cells /μl, 2) capacity of T cell proliferation, 3) ratio of $CD4^+$ T cells/$CD8^+$ T cells, 4) number of $CD4^+$ naïve T cells, 5) ratio of $CD4^+$naïve T cells/ $CD4^+$memory T cells, 6) number of B cells, 7) number of NK cells, 8) IL-2 production, 9) IFNγ production,10) IL-4 production. Each index was given a score from 1 to 3 (low, medium and high) based upon the data base and the summation of total scores was named SIV and used as the estimated grade of immunological vigor of individuals, ranging between 10 and 30. SIV of healthy people ranged between 23 and 30. SIV of cancer patients ranged in the lower zone between 26 and 16. The correlation equation is quite different between these two groups. In cancer patients, SIV rapidly declines with age (Figure-1).

SIV was classified into 5 grades; grade 5 means that immunological vigor is well preserved. Grade 4 is fairly good. Grade 3 is borderline and in the initial phase of decline. Grade 2 is moderate decline and Grade 1 is severe decline (Figure-2).

Most healthy people belonged to grades 4 and 5. Several people belonged to grade 3. It is interesting to note that half of all cancer patients were still in the range of grades 4 and 3, but one third were already in grades 2 and 1.

In our opinion, people with grade 2 or 3 should be requested to consider the restoration of immunological vigor by some method. People with grade 1 should be treated with rapidly effective methods for immunological restoration or at least be kept in a clean room.

Assessment of the immunological state of individual people is now necessary. Just like blood pressure, blood glucose level, HbA1c or liver functions, people should know their own immunological state, high, medium or low. Assessment of the immunological state is especially important for patients suffering from various diseases, because they undergo various treatments that may suppress immune functions. The next point is the solution for a depressed immunological state. The issue is how to restore or reconstruct immunological functions.

3. Restoration of the immunological state.

The following is a list of experiments of immunological restoration previously conducted in my laboratory using animal models (5,6).

a) Activation of existent immune cells.
   1. Anti-oxidants such as VE.

2. Low dose anti-cancer drugs.
3. Caloric restriction.
4. Exercise.
5. Vaccination.
6. Japanese herbal medicine

b) Grafting of tissue or cells
   1. Combined grafting of BM and newborn thymus.
   2. Grafting of newborn thymus.

c) Infusion of activated autologous/syngeneic T cells.

In this presentation, I can not explain all of these methods in detail. Briefly speaking, they have all, except for grafting newborn thymus, been more or less applied to humans with a certain degree of effect. In my understanding, the proper assessment of whole immunological functions is necessary for any immunological restoration. The effectiveness of restorative methods may differ by individuals and one has to select the most effective method by immunological monitoring (Figure-3). Scoring of immunological vigor (SIV) shown in this presentation could be useful for the immunological monitoring. Currently, the most effective method of immunological restoration is the infusion of activated T cells propagated in vitro using lymphocytes of an individual. This method has been widely used for cancer patients as immunotherapy (7), but without significant cure of cancer in many cases.

T cells of patients with immune deficiency can not efficiently proliferate in vivo by the usual antigenic stimulation. These T cells, however, can be expanded ex vivo, in the presence of immobilized anti-CD3 Ab and IL-2. However, ideal source of T cells for in vitro culture is those obtained from healthy individuals. T cells obtained from individuals at young age or healthy period can be stored in liquid nitrogen for many years. When needed, frozen T cells can be thawed and propagated ex vivo for the infusion. The infusion of autologous activated T cells propagated ex vivo is useful for the treatment of children with congenital immune deficiency (9), so why not for elderly people. Fortunately, a recent paper reported that immunotherapy with activated T cells propagated in this way did not enhance or promote autoimmune phenomena (8). One important point for the infusion of autologous activated T cells is that we need T cell bank system where one can store healthy and active T cells in liquid nitrogen for many years and can use these T cells when necessary.

4. Effect of infusing activated T cells into a mouse model.

We observed the effect of infusing activated T cells using young and old mice (10). In this experiment, we employed a congenic combination of B10.Thy-1.1 mice as donors and C57BL/6 Thy-1.2. mice as recipients.

After the infusion of activated T cells, the absolute number of T cells increased in the spleen of recipient mice, especially aged mice. In the peripheral blood and spleen, donor-type T cells were significantly more numerous in the aged recipients than in young ones. In addition, many more donor-type T cells were present in the spleen than in peripheral blood in both young and old recipients.

The magnitude of antibody formation against SRBC did not change significantly in young recipients. In aged recipients, however, it is interesting to note that significant enhancement of antibody formation was observed in more than half of recipients, even in the aged recipients infused with activated T cells from aged donors.

Conclusion

1) Decline of immunological functions is caused by various factors such as aging, stress, therapy for disease, inappropriate lifestyle, etc and give rises to decline of QOL and deterioration of diseases.
2) In order to know immunological status, proper assessment of immunological functions is urgently needed, especially for the elderly and patients suffering from various diseases. This presentation has proposed one method to assess the immunological state as a whole by scoring immunological vigor.
3) Immunological restoration should be tried, if necessary, after the assessment of immunological functions.
4) For human application, infusion of autologous activated T cells could be used for immunological restoration.
5) A T cell bank system could be useful for infusion of autologous activated T cells.

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Figure- 1 SIV in healthy people and cancer patients.

Figure 2:SIV grade in healthy and cancer patients

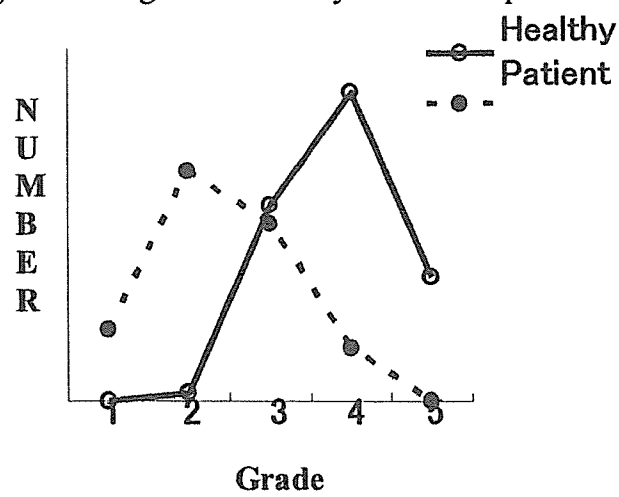

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Figure-3 Immunological restoration under immunological monitoring

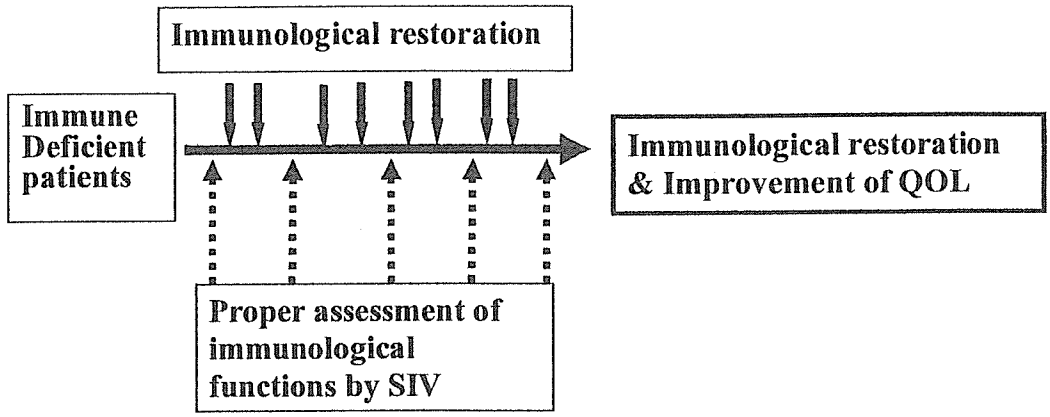

Table-1 Infection is top cause of death in the elderly

Bronchopneumonia	***43.9 %***
Malignant neoplasms	**28.1 %**
Pulmonary thrombo-embolism	**21.2 %**
Acute myocardial infarction	**19.6 %**
Urinary tract infection	***12.3%***
Acute cerebrovascular disease	**6.5 %**
Internal hemorrhage	**5.5 %**
Congestive cardiac failure	**3.3 %**

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Cell Injury, Repair, Aging and Apoptosis

Overexpression of Peroxisome Proliferator-Activated Receptor γ Co-Activator-1α Leads to Muscle Atrophy with Depletion of ATP

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Peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) is a key nuclear receptor co-activator for mitochondrial biogenesis. Here we report that overexpression of PGC-1α in skeletal muscles increased mitochondrial number and caused atrophy of skeletal muscle, especially type 2B fiber-rich muscles (gastrocnemius, quadriceps, and plantaris). Muscle atrophy became evident at 25 weeks of age, and a portion of the muscle was replaced by adipocytes. Mice showed increased energy expenditure and reduced body weight; thyroid hormone levels were normal. Mitochondria exhibited normal respiratory chain activity per mitochondrion; however, mitochondrial respiration was not inhibited by an ATP synthase inhibitor, oligomycin, clearly indicating that oxidative phosphorylation was uncoupled. Accordingly, ATP content in gastrocnemius was markedly reduced. A similar phenotype is observed in Luft's disease, a mitochondrial disorder that involves increased uncoupling of respiration and muscle atrophy. Our results indicate that overexpression of PGC-1α in skeletal muscle increases not only mitochondrial biogenesis but also uncoupling of respiration, resulting in muscle atrophy. ***(Am J Pathol 2006, 169:1129–1139; DOI: 10.2353/ajpath.2006.060034)***

Peroxisome proliferator-activated receptor (PPAR)-γ co-activator-1α (PGC-1α), which was identified as co-activator of nuclear receptors, is expressed in brown adipose tissue, skeletal muscle, heart, kidney, and brain and is markedly up-regulated in brown adipose tissue and skeletal muscle after acute exposure to cold stress.[1] Increased expression of PGC-1α as part of activated adaptive thermogenesis occurs primarily in the mitochondria of brown adipose tissue and skeletal muscle through stimulation of mitochondrial biogenesis and respiration.[2] PGC-1α is also believed to be a key molecule involved in fatty acid oxidation because it was found to interact with PPARα to promote transcription of nuclear genes encoding mitochondrial fatty acid oxidation enzymes.[3,4] In *in vivo* studies of muscles overexpressing PGC-1α, the skeletal muscles showed a red color characteristic of oxidative muscle and elevated levels of enzymes related to mitochondrial oxidative phosphorylation and fatty acid oxidation.[5] These same muscles showed reduced GLUT4 mRNA expression and impaired insulin tolerance.[6] In brown adipose tissue mitochondria, as observed during prolonged exposure to cold, PGC-1α promotes uncoupling of respiration through induction of uncoupling protein 1 (UCP1).[1] When respiration is uncoupled, the membrane potential energy is channeled to heat production rather than to adenosine triphosphate (ATP) production, which may cause functional abnormalities and cell death. In C2C12 myocytes, increased uncoupling of respiration driven by PGC-1α was observed.[7] Overexpression of PGC-1α promoted mitochondrial biogenesis and resulted in dilated cardiomyopathy.[4] These data suggest that PGC-1α overexpression in skeletal muscles increases uncoupling of respiration, decreases

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