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EFFECTS OF HYPERLIPAEMIC SERUM ON INTERLEUKIN-2 (IL-2) PRODUCTION, IL-2 RECEPTOR EXPRESSION, AND T CELL PROLIFERATION INDUCED BY IL-2 IN CYNOMOLGUS MONKEYS

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SUMMARY: The effect of hyperlipaemic serum on mitogen-induced T lymphocyte proliferation was investigated with cynomolgus monkeys. The mitogen-induced blastogenesis was remarkably inhibited when either hyperlipaemic or normal monkey lymphocytes were incubated with hyperlipaemic sera. Hyperlipaemic serum also inhibited ConA-induced interleukin 2 (IL-2) production as well as IL-2 receptor (IL-2R) expression of normal monkey lymphocytes. On the other hand, it showed slight inhibition of T-cell proliferation induced by adding recombinant human IL-2 to IL-2R-positive normal monkey lymphocytes. These results indicate that hyperlipaemic serum inhibited an early stage of T-cell autocrine activation pathway including IL-2 production and IL-2R expression.

INTRODUCTION

Epidemiologic and clinical data suggest that obesity or obesity-related metabolic disorders in man such as hypercholesterolemia and adult diabetes

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mellitus are associated with alteration or impairment of immune functions, resulting in an increased vulnerability to various infectious diseases or cancer (1). However, the effect of severe obesity on humoral or cell-mediated immunity is still controversial in human (2,3).

We have demonstrated that the lymphocytes of obese and/or hyperlipaemic cynomolgus monkey showed the normal mitogen-induced blastogenesis, but hyperlipaemic serum could inhibit blastogenesis of not only autologous lymphocytes but also lymphocytes of unrelated normal monkeys (unpublished data). T-lymphocyte (T cell) proliferation requires interleukin 2 (IL-2) production, IL-2 receptor (IL-2R) expression and IL-2 binding to IL-2R, which complete the autocrine T-cell proliferation (4,5). Therefore, it seems necessary to determine which stage of T-cell proliferation is affected by hyperlipaemic sera. This paper deals with the effects of hyperlipaemic serum on T-cell proliferation including IL-2 production, IL-2R expression, in obese and hyperlipaemic cynomolgus monkeys as a model of human cases.

MATERIALS AND METHODS

Animals: Blood specimens were collected from two obese and hyperlipaemic female cynomolgus monkeys (*Macaca fascicularis*; Nos. 182047 and 182020) aged 7 years. As to normal control, blood specimens were collected from seven age-matched female cynomolgus monkeys whose clinical and biochemical data were within normal limits (6). Three healthy male monkeys aged 5 to 7 years were used as sources of the peripheral blood mononuclear cells (PBMC). In addition, sera collected from 20 healthy young male cynomolgus monkeys were used as pooled normal serum (pool serum). All animals used were bred and reared at the Tsukuba Primate Center for Medical Science (TPC), National Institute of Health (7). The environmental conditions of the breeding facility were described elsewhere (7). They were each fed 100 g of oranges and apples in the morning and 70 g of monkey chow (Type-AS, Oriental Yeast, Tokyo) in the afternoon everyday.

To judge the obesity of the monkey, we employed the cynomolgus body-mass index (CyBMI) calculated by the following formula:

$$\text{CyBMI} = \text{Body weight (kg)} / \text{Sitting height (m)}^2$$

Lymphocyte separation and mitogen-induced blastogenesis: Blood specimens were collected between 10:00 to 10:30 a.m. after 16-hr fasting. A 10-ml portion of heparinized blood was mixed with 5 ml of phosphate buffered saline

(PBS, pH 7.6) containing 5% of dextran (Dextran 2000, Pharmacia, Uppsala, Sweden) and the mixture was incubated at 37 C for 30 min. Then, the supernatant was collected and centrifuged at 2,000 rpm for 10 min. The cell pellet was resuspended in PBS and PBMC were isolated by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation. The PBMC were washed three times with PBS and suspended in RPMI-1640 medium (Flow, Scotland) supplemented with 2% fetal calf serum (FCS, Gibco, Gland Island, NY) at a cell density of $2.5 \times 10^6/\text{ml}$. The viability of separated PBMC was greater than 95% as determined by trypan blue exclusion. A reaction mixture (200 μl) for blastogenesis contained 2×10^5 of PBMC, 20% of autologous, hyperlipaemic or pool serum and 10 $\mu\text{g}/\text{ml}$ (final) of phytohemagglutinine (PHA: Sigma, St. Louis, MO) or concanavarin A (ConA: Sigma). They were mixed in a well of a culture plate (Falcon, Oxnard, CA) and incubated at 37 C under 5% CO_2 -air condition. After 3-day culture, 1 μCi of ^3H -thymidine (TdR: Amersham Corp., Arlington, IL) was added to each well and the culture was pulsed for 16 hr. The lymphocytes were harvested onto glass fiber filters and determined for TdR incorporation with a liquid scintillation counter (Packard, Rockville, MD).

IL-2 production and determination of IL-2 contents: IL-2 production by PBMC was examined by the method previously established (unpublished data). Briefly, 500 μl of a culture mixture containing 5×10^5 of PBMC, 20% of hyperlipaemic or pool serum and 10 $\mu\text{g}/\text{ml}$ of ConA were put in a 48-well culture plate (Falcon) and incubated at 37 C under 5% CO_2 -air condition. After 48 hr, the culture supernatant was collected and stored at -80 C until IL-2 assay. The IL-2 contents in culture supernatant were determined by the growth of IL-2-dependent mouse cell line (HT-2 cells) which was kindly given by Dr. M. Honda (AIDS Research Center, NIH, Japan). The supernatant was diluted 1:2 with RPMI medium supplemented with 10% FCS and 5 mM 2-mercaptoethanol. A 100- μl portion of the diluted supernatant was mixed with 100 μl of a HT-2 cell suspension which contained 4×10^3 cells. The mixtures were incubated for 24 hr and 0.5 μCi of TdR was added 6 hr before harvesting cells. The IL-2 content in supernatant was expressed as TdR incorporation (cpm) / 4×10^3 HT-2 cells.

Measurement of IL-2 receptor-positive PBMC: The IL-2R-positive cells were induced by culturing 1×10^6 cells with 10 $\mu\text{g}/\text{ml}$ of ConA in the presence of 20% of hyperlipaemic or pool serum. After 3 days of culture, 100 μl of the cultured cell suspension was mixed with 10 μl of an anti-human IL-2R α (TA-75) or anti-human IL-2R β (Mik β -1) (8) monoclonal antibody, both of which react with cynomolgus monkey IL-2R (unpublished data). The mixtures were incubated for 45 min on ice and washed twice with RPMI-1640 containing 2% FCS and 0.1% sodium azide (FACS media), and then, 10 μl of diluted (1:40) FITC-anti-mouse IgG antibody (Cappel, Cochranville, PA) was added to each sample. The mixtures were allowed to stand for 30 min on ice and washed twice with FACS media. The stained cells were fixed with 200 μl of a buffered formalin solution (1% paraformaldehyde in PBS) and kept at 4 C until FACS analysis.

The FACS analysis was performed with a FACScan Cell Sorter (Becton Dickinson, Paramus, NJ) with forward- and right-angle scatter gates set on the lymphocytes including proliferating cells (Fig. 1). For each sample, data from 10,000 cells were collected and the result of analysis was expressed as percentages of IL-2R α - and β -positive cells to total lymphocytes.

T-cell proliferation induced by binding human recombinant IL-2 to IL-2R-positive normal monkey lymphocytes: To induce IL-2R-positive cells, 1×10^7 of normal monkey PBMC were incubated with 10 $\mu\text{g/ml}$ of ConA in 5 ml of RPMI medium supplemented with 10% of pool serum at 37 C for 3 days. The cultured cells were collected, washed three times with 1% FCS-PBS and resuspended in RPMI medium at a cell density of $2 \times 10^6/\text{ml}$. A 100- μl portion of the cell suspension was mixed with 40 μl of hyperlipaemic or pool serum (final: 20%) in a culture plate (Falcon). Then, 60 μl of recombinant human IL-2 (rh-IL-2; Fujisawa, Osaka) was added to a final concentration of 100 IU/ml or 10 IU/ml. The mixtures were incubated at 37 C for 2 days and one μCi of TdR was added 16 hr before harvesting cells onto a glass fiber filter. IL-2-dependent T-cell proliferation was expressed by Δcpm calculated by subtracting TdR incorporation (cpm) in culture with medium alone from that in culture with rh-IL-2.

Every reaction was carried out in triplicate.

RESULTS

Mitogen-induced Lymphocyte Blastogenesis of Hyperlipaemic Monkeys

Table I shows the clinical and biochemical parameters of the two obese and hyperlipaemic monkeys. We calculated CyBMI by body weight (kg) and sitting height (m)² and judged monkeys whose CyBMI was more than 27.5 as obese. Monkeys showing more than 148 mg/dl of total cholesterol content or more than 81 mg/dl of triglycerides concentration were judged as hyperlipaemic (unpublished data). As shown in Table I, the female monkeys (Nos. 182047 and 182020) were judged as obese and hyperlipaemic. Table II shows the mitogen-induced blastogenesis of two hyperlipaemic monkey PBMC determined by two different culture conditions, culture with autologous serum or pool serum. Normal monkey PBMC showed no difference in blastogenesis between the two different culture conditions. However, the blastogenesis of two hyperlipaemic monkey PBMC was significantly lower in the culture with autologous serum than in the culture with pool serum. Figure 1 shows the scatter analysis of normal monkey lymphocytes after 3-day culture with 10 $\mu\text{g/ml}$ of ConA in the presence of 20%

Table I. Clinical and biochemical parameters of two obese and hyperlipaemic monkeys

	182047	182020	Normal monkeys (N:7)#
Body weight (kg)	6.1	6.4	4.0 ± 1.4
CyBMI##	36.8	36.7	21.4 ± 4.1
Total cholesterol (mg/dl)	164	159	112.2 ± 16.4
HDL cholesterol (mg/dl)	30	35	54.7 ± 7.6
LDL + VLDL (mg/dl)	134	124	57.5 ± 10.5
Triglycerides (mg/dl)	94	106	72.6 ± 14.4

#: Normal monkeys: healthy age-matched seven female monkeys used as control.

##: CyBMI (cynomolgus monkey body-mass index) = Body weight (kg) / Sitting height (m)².

pool serum (A) or two hyperlipaemic monkey sera (B and C). The proliferating cells were clearly determined by flow cytometry with forward-angle light scatter (FSC) versus right-angle side scatter (SSC). The proliferating cells were significantly less in the culture with hyperlipaemic serum (B and C) than in the culture with pool serum (A).

ConA-induced IL-2 Production and IL-2R Expression

Effects of hyperlipaemic serum on blastogenesis, IL-2 production and IL-2R expression are shown in Table III. PHA- or ConA-induced blastogenesis of normal monkey lymphocytes was significantly inhibited by culturing with hyperlipaemic serum. Each hyperlipaemic serum inhibited ConA-induced IL-2 production as well as IL-2R expression by normal monkey lymphocytes. IL-2 contents determined by TdR incorporation in HT-2 cells were significantly lower in culture with hyperlipaemic sera than those in culture with pool serum. The FACS profiles of IL-2R α - and β -positive cells are shown in Fig. 2. Both IL-2R α and β -positive cells decreased in number in the culture with hyperlipaemic serum (B and C) as compared with that in the culture with pool serum (A). The inhibitory effects of hyperlipaemic serum on IL-2R expression were remarkable

Table II. Mitogen-induced blastogenesis of obese and hyperlipaemic monkey lymphocytes

	182047	182020	Normal monkeys (N:7)#
PHA-induced blastogenesis with autologus serum	163,449 **	25,406 **	140,851 ± 41,880# (Range:229,260–96,572)
pooled allo serum	250,335	84,843	181,381 ± 73,235 (Range:270,181–92,581)
ConA-induced blastogenesis with autologus serum	165,561 **	48,488 *	178,061 ± 60,883 (Range:253,571–68,396)
pooled allo serum	222,825	63,774	140,863 ± 61,826 (Range:207,858–73,867)

Data were expressed by TdR incorporation (cpm) / 2×10^5 PBMC.

*: $p < 0.05$, **: $p < 0.01$.

#Mean ± SD of responses of seven age-matched normal monkey PBMC.

in IL-2R α expression (Fig. 2 and Table III). The percentages of IL-2R α - and β -positive cells in the culture with hyperlipaemic sera were 15 to 30% and 50% of those in the culture with pool serum, respectively.

The Effects of Hyperlipaemic Sera on T-Cell Proliferation Induced by rh-IL-2

T-cell proliferation induced by binding rh-IL-2 to IL-2R-positive cells are shown also in Table III. Each hyperlipaemic serum showed slight inhibition on T cell proliferation in the culture with rh IL-2. A statistically significant decrease was observed only in the cultures with 100 IU/ml and 10 IU/ml of rh-IL-2 in the presence of 182020 and 182047 serum, respectively.

Fig. 1. Scatter analyses of ConA-induced proliferating cells.
 A: Cultured with 20% of pool serum.
 B: Cultured with 20% of No. 182047 serum.
 C: Cultured with 20% of No. 182020 serum.

DISCUSSION

Suppressive effects of malnutrition on immune functions have been widely discussed. In fact, it has been reported that malnourished human population showed marked depression of both cell-mediated and humoral immunities (9,10). On the other hand, effects of overnutrition on the host-defense mechanisms are

Fig. 2. FACS profiles of two kinds of IL-2 receptor-positive cells.

A: Cultured with 20% of pool serum.

B: Cultured with 20% of No. 182047 serum.

C: Cultured with 20% of No. 182020 serum.

still unclear. As to human obesity caused by overnutrition, severe overweight itself has generally been accepted to have no significant influence on major immunologic functions (3). Some investigators, however, reported that variable impairments of cell-mediated immune response were noticed in obese children (2). Actually, obese humans are different from one to another in their life histories, age, nutritional states and obesity-associated metabolic disorders (11). Therefore, it is rather difficult to draw a definite conclusion concerning the effects of overnutrition on the immune function on the basis of data from human subjects.

Spontaneously occurring or experimentally induced obesity, obesity-associated metabolic disorder such as hypercholesterolemia and diabetes mellitus have been reported in several nonhuman primate species (12,13). Laboratory primates must be a suitable animal model for determining effects of obesity or of obesity-associated metabolic disorders on immune functions because the environmental conditions including dietary regimen can strictly be controlled

Table III. The effects of hyperlipaemic serum on blastogenesis, IL-2 production, IL-2 receptor expression and T cell proliferation induced by binding of IL-2 to IL-2 receptor

Responses (mean ± SD) of three normal monkey PBMC in culture with			
	182047 serum	182020 serum	Pooled allo serum
Blastogenesis in culture with			
Medium (cpm)	1,165 ± 311	950 ± 334	1,043 ± 348
PHA (cpm)	2,022 ± 526**	36,999 ± 27,974**	86,987 ± 26,989
ConA (cpm)	6,367 ± 8,107**	40,475 ± 29,131**	172,236 ± 36,676
ConA-induced IL-2 production (cpm/4 × 10 ³ HT-2 cells)	20,340 ± 10,740**	30,019 ± 17,982*	54,356 ± 12,035
IL-2R expression			
% of IL-2R α ⁺ cells#	14.8 ± 5.5*	16.1 ± 9.3*	59.5 ± 25.4
% of IL-2R β ⁺ cells	31.7 ± 10.9**	36.7 ± 15.8*	62.9 ± 4.6
T cell proliferation (Δ cpm)			
induced by rhIL-2 (100 IU)#	126,489 ± 25,904	124,492 ± 37,801*	144,895 ± 37,667
rhIL-2 (10 IU)	61,530 ± 5,302*	62,300 ± 13,031	80,435 ± 10,826

** : P < 0.01, * : P < 0.05 as compared with response in culture with pool serum.

: Percentages of IL-2R-positive cells to total PBMC.

##rhIL-2: Recombinant human IL-2.

with artificial housing, feeding and breeding procedure (12). Our previous survey revealed that about 15% of cynomolgus monkeys were classified into obese and/or hyperlipaemic in our breeding colony, although they were housed under the same conditions (unpublished data). In addition, our previous study with 15 obese and/or hyperlipaemic cynomolgus monkeys demonstrated that the mitogen-induced lymphocyte blastogenesis was normal in these monkeys but hyperlipaemic serum inhibited blastogenesis of normal monkey lymphocytes (unpublished data). These results suggest that simple obesity is not related to immunologic dysfunction but obesity-associated hyperlipaemia affects T-cell response in cynomolgus monkeys. The autocrine activation of T cells requires IL-2 production, IL-2R expression and IL-2 binding to high-affinity IL-2R, which complete the activation cycle (4,5). The question is which stage of T-cell activation pathway is affected by hyperlipaemic serum in cynomolgus monkeys. In this study, we demonstrated that two hyperlipaemic monkey sera each significantly inhibited both mitogen-induced IL-2 production and IL-2R expression. This result suggests that hyperlipaemic serum influence early stage in mitogen-induced T cell activation. There is a possibility that hyperlipaemic serum itself has some cytotoxic effect on lymphocytes, thus resulting in lower T-cell proliferation. However, this may be ruled out by the fact that there was no definite difference between cultures with pool serum and that with hyperlipaemic serum in IL-2-dependent T-cell proliferation (Table III).

The mitogen-induced T-cell activation can be triggered by at least two signals; mitogen-induced transmembrane signal and IL-2 (14). The mitogen-induced primary signal involves a number of changes in membrane lipids, resulting in transforming T cells from a resting 'G0' phase to a 'G1' phase of the cell cycle (14). These primary changes of membrane lipid occur within few minutes after mitogen stimulation. In addition, it was presumed that excess cholesterol increased the cell membrane cholesterol contents and altered the membrane fluidity, resulting in change in the immune functions (15). Thus, the effects of hyperlipaemic serum on the transmembrane components can point out its important role in transforming the resting T cells to primed ones, which induce IL-2 production and IL-2R expression. However, the precise mechanisms of inhibition of T-cell proliferation by hyperlipaemic sera should further be scrutinized with each of transmembrane signals with purified serum cholesterol or triglycerides.

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