Inhibition of IL-2 production by Nil-2-a in murine T cells

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Abstract

The loss of IL-2 production is the main defect accounting for age-related immunodeficiencies. We have investigated the molecular mechanisms involved in the decrease of IL-2 production in CD4\(^+\) T cells from aging mice. Our results demonstrate that the stability of IL-2 mRNA increases in T cells from young mice, whereas it declines in T cells from old mice with the time of stimulation, suggesting the existence of different mechanisms of post-transcriptional regulation in young and old mice. We found that the IL-2 mRNA level in T cells from young but not from old mice increased up to 6- to 10-fold by addition of cycloheximide (CHX) while the stability of IL-2 mRNA is not affected. We then looked for IL-2 inducible inhibitory factors in T cells from young and old mice and demonstrated the presence of Nil-2-a, a zinc finger protein which negatively controls IL-2 gene transcription in human cells. This protein could be detected in T cells from both young and old mice, yet, in the presence of CHX, its binding activity was reduced by 75% in T cells from young but not from old mice. These findings show that Nil-2-a accounts for the negative control of IL-2 production in the mouse and explain the reduced IL-2 production in aging.

Introduction

Aging is characterized by a progressive decline in the capacity to respond to exogenous antigens and an increase in the production of autoantibodies (1). The mechanisms of age-related immune dysfunctions are not well known. There is general consensus, however, on the decrease in the expression of receptors for growth-promoting factors (2) and on the alteration in signal transduction pathways (3–5) leading to dysregulation in cytokine production (6–9). As to IL-2, several results have demonstrated that aging negatively affects IL-2 production regardless of the stimulus used (10).

It is known that the level of IL-2 mRNA in lymphocytes is dynamically regulated by the rates of synthesis and degradation, the latter rate being markedly influenced by the state of cell activation (11). In the present paper, we have investigated the molecular mechanisms involved in IL-2 production in lymphocytes from young and old mice.

Methods

Animals

Female C57BL/6 mice, bred and maintained in our animal facilities, were used at the age of 3 (young) or 19–20 (old) months. These mice exhibited a mean survival time of ~750 days and display age-related T cell deficiencies as early as 6 months from birth. In each experiment, splenocytes were pooled from 10–30 mice/group.

Positive selection of splenic T CD4\(^+\) cells

Mice were killed by decapitation, their spleens aseptically removed and single-cell suspensions prepared. Red blood cells were removed by osmotic shock. Briefly, pelleted spleen cells were resuspended in 1 ml of 0.1×PBS, in agitation. After 15 s, 1 ml of 2×PBS was added for an additional 15 s, in agitation. Cells were then washed with 1×PBS. Macrophages were depleted by plastic adherence during 1 h of incubation in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 5% FCS (Flow, Irvine, UK), at 37°C in a 5% CO\(_2\) humidified incubator. Non-adherent cells were then washed and resuspended in 1×PBS, incubated (10^8 cells/ml) for 20 min on ice with mouse anti-CD4 (L3T4) MicroBeads (1:10, v/v) (492-01; Miltenyi Biotech, Germany), according to the MiniMacs protocol. Briefly, 100 µl of MicroBeads was used for 10^7 cells. After incubation with MicroBeads, cells were washed and resuspended in 500 µl PBS, and applied to the top of a...
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Prefilled and washed A2 column (Miltenyi), provided with a 2 ml of PBS and, after removal of the needle, withdrawn from the magnetic field. Labeled cells were eluted from the column with 1 ml PBS and found to be almost exclusively (>98%) CD4+ by cytofluorimetric analysis. Eluted cells represented 20% of the initial spleen cell population.

Cell culture

T CD4+ cells were cultured in complete medium consisting of RPMI 1640, supplemented with 5% FCS, 2 mM L-glutamine (Gibco), 10 µg/ml gentamicin (Shering, Kenilworth, NY) and 5x10^-5 M 2-mercaptoethanol, as described (12). Cells were cultured for different times (3-40 h) in tissue culture plates (3047, Falcon, Oxnard, CA), pre-coated with 10 µg/ml of anti-CD3ε mAb (clone 145-2C11) and with 1 µg/ml of soluble anti-CD28 mAb (clone 37.5G1; PharMingen, San Diego, CA).

Cytokine determination in culture supernatants

IL-2 production was measured by ELISA assay. Briefly, wells of microtiter plates (3590; Costar, Cambridge, MA) were coated (overnight, 4°C) with 50 µl of a purified rat anti-mouse IL-2 antibody (18161D; PharMingen) at a concentration of 1 µg/ml in PBS. Reactions were blocked by adding to each well 200 µl of PBS containing 0.5% bovine casein (C7078; Sigma, St Louis, MO) for 2 h at room temperature. Wells were then washed thoroughly with PBS and then received (50 µl/well) the detecting antibody (biotin-conjugated, 18172D; PharMingen) at a concentration of 1 µg/ml in PBS. Reactions were blocked by adding to each well 50 µl/well of ABTS (1 volume of peroxidase solution of H2O2) and 1 µl/well of avidin–peroxidase (A3151; Sigma) in WB buffer. After 1 h incubation at room temperature, washed wells received 75 µl of avidin–peroxidase (A3151; Sigma) in WB and incubated for 1 h at room temperature. Washed wells then received 100 µl/well of ABTS (1 volume of peroxidase substrate + 1 volume of peroxidase solution of H2O2) and were incubated for 15–20 min at room temperature. Reactions were blocked by addition of 50 µl/well of an 0.2 M solution of citric acid. Well contents were measured for absorbance at 405 nm.

Preparation of cDNA and RT-PCR

Total RNA samples were prepared from CD4+ cells using guanidine isothiocyanate lysis (13). A 1 µg aliquot of each RNA sample was reverse transcribed and RT-PCR reactions were performed as follows. The cDNA was amplified in a DNA thermocycler (9600; Perkin-Elmer, Norwalk, CT). Briefly, reaction mixtures consisted of 2 µl of reverse transcribed RNA in 20 µl of RT buffer containing 1.2 µM of each primer, 0.3 µl of 25 mM dNTP and 1.2 U of ampliqaq polymerase. Thirty cycles were performed under the following conditions: 1 min at 94°C, 30 s at 60°C and 1 min at 72°C. The number of cycles was determined according to the necessity to avoid reaction saturability. Reactions were performed in duplicate. The amount of cytokine-specific mRNA has been referred to the amount of β-actin mRNA. The primers specific for murine IL-2 and β-actin, used for PCR analyses, have been previously described (14). To evaluate mRNA stability, CD4+ cells were activated with anti-CD3 and anti-CD28 mAbs for different times of culture and then mRNA synthesis was blocked by Actinomycin D (10 µg/ml). After 15, 45 and 90 min, RNA samples were collected and extracted as described above. In order to block protein synthesis, cycloheximide (CHX) was added in the last 2 h of culture, at a concentration of 10 µg/ml.

Preparation of nuclear extracts

CD4+ cells (1x10^7) were pelleted by centrifugation at 1500 g for 5 min. The pellet was resuspended in 50 µl of cold buffer A (HEPES 10 mM, pH 7.9, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1 mM and PMSF 0.5 mM), by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 µl of a 10% solution of Nonidet NP-40 (Fluka) was added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microfuge. The nuclear pellet was resuspended in 50 µl of ice-cold buffer C (HEPES–KOH 20 mM, pH 7.9, glycerol 25%, NaCl 420 mM, MgCl2 1.5 mM, EDTA 0.2 mM, DTT 0.5 mM and PMSF 0.2 mM) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C. The supernatant fraction, containing DNA binding proteins, was stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

A 32P-end-labeled 24 bp DNA probe, which included the –112 to –99 negative regulatory element (NRE-A) domain (5’-AAT TCC AGA CAG GTA AAG TGT TAA-3’) (15), was incubated with nuclear extracts prepared from CD4+ cells which were either resting or activated. Briefly, DNA binding reactions were incubated for 30 min at 23°C and contained labeled probe (1 ng), nuclear extracts (2 µg), poly(dI–dC) (3 µg), glycerol (12%), HEPES (12 mM, pH 7.9) and KCl (60 mM). In oligonucleotide competition experiments, a 10-fold excess of the unlabeled oligonucleotide was added. Complexes were separated on polyacrylamide gel in TBE (45 mM Tris–borate, 1 mM EDTA, pH 8.0).

Results

Kinetics of IL-2 mRNA accumulation and expression

Results in Fig. 1 are from one of four experiments. Figure 1(A) shows that after 3 h of culture there is no difference in the amount of IL-2 mRNA in T CD4+ lymphocytes from young and old mice. At 16 h of culture, T CD4+ cells from old mice display a higher amount of IL-2 mRNA (2-fold increase) as compared to CD4+ cells from young mice. In another three experiments, the differences between young and old mice were the following: 2-fold increase in experiment 1, 2.4-fold increase in experiment 2 and >10-fold increase in experiment 3. Conversely, at 19 h, CD4+ cells from young and old mice exhibited comparable levels of IL-2 mRNA. Thus, mRNA accumulation displays a faster rate in cells from young than in cells from old mice, the increase being 17-fold in the former group and 10-fold in the latter during the overall stimulation time of 3–19 h. The kinetics of mRNA expression in culture supernatants of T CD4+ cells was studied after 16, 24 and 40 h of stimulation. The experiment reported in Fig. 1(A) has also been performed using a Northern blot analysis. Figure
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Fig. 1. IL-2 expression in CD4⁺ cells from young and old mice at different times of stimulation. (A) Scanning densitometric analysis of IL-2 mRNA levels in CD4⁺ cells stimulated with anti-CD3 and anti-CD28 mAbs for 3 (white), 16 (dashed) and 19 (shadow) h. (B) Kinetics of the accumulation of IL-2 protein, determined by ELISA, in culture supernatants. Young (black circles); old (white circles).

Fig. 2. Effect of the stimulation time on the rate of IL-2 mRNA degradation in CD4⁺ cells from young and old mice. The amount of mRNA at various times is expressed as a fraction of the mRNA level at time 0. The amount of mRNA was determined by scanning densitometry. Values are the mean of three independent experiments. Young (black symbols); old (white symbols). Triangles, 3 h of stimulation; circles, 16 h of stimulation; squares, 16 h of stimulation.

1(B) shows a progressive increase in IL-2 concentration with the time of stimulation in young mice, whereas in old mice it first increased and then decreased, being comparable to the level observed in young mice at 19 h but below at 40 h of stimulation. Thus, the amount of mRNA positively correlates with the IL-2 concentration in young but not in old mice. Since mRNA stability may change with aging, this issue is addressed in the following section.

Effect of the stimulation time on the stability of IL-2 mRNA
Results in Fig. 2 show that the stability of IL-2 mRNA in T CD4⁺ cells from young mice progressively increased with the increasing time of stimulation. Conversely, the stability of IL-2 mRNA in T CD4⁺ cells from old mice progressively decreased with the increasing time of stimulation, suggesting the existence of different mechanisms of post-transcriptional regulation in T CD4⁺ lymphocytes from young and old mice. Thus, the reduced stability of IL-2 mRNA in old mice may explain the different kinetics of mRNA accumulation and expression. To investigate the mechanisms involved in post-transcriptional regulation of IL-2 mRNA in young and old mice, mRNA translation was blocked by CHX.

Effect of CHX on the accumulation and stability of IL-2 mRNA
Cultures were activated for 1 or 17 h and CHX was then added for an additional 2 h of stimulation. It was found that after a total of 3 h stimulation there was no significant difference in the accumulation of IL-2 mRNA between cultures of CD4⁺ cells from old mice stimulated in the presence or absence of CHX, whereas IL-2 mRNA from culture of CD4⁺ cells from young mice was slightly higher in the absence of CHX as compared to CHX-treated cultures (data not shown). Conversely, Fig. 3 shows that after a total of 19 h of stimulation, CHX induced a 6- to 10-fold increase in the accumulation of IL-2 mRNA.
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**Fig. 3.** IL-2 mRNA levels in CD4\(^+\) cells from young (A) and old (C) mice after 19 h of stimulation. Effect of CHX on the accumulation of IL-2 mRNA in CD4\(^+\) cells from young (B) and old (D) mice. (A’–D’) β-actin. The experiment was repeated with similar results.

**Fig. 4.** Effect of CHX on the stability of IL-2 mRNA in CD4\(^+\) cells from young and old mice after 19 h of stimulation. Results are expressed as in Fig. 2 and are from three independent experiments. Triangles, 19 h stimulated CD4\(^+\) cells from young mice, in the presence (black) or absence (white) of CHX. Squares, 19 h stimulated CD4\(^+\) cells from old mice, in the presence (black) or absence (white) of CHX.

IL-2 mRNA in CD4\(^+\) cells from young but not from old mice (<2-fold induction).

In order to examine whether CHX also had an effect on the stability of IL-2 mRNA, we performed an experiment in which CD4\(^+\) lymphocytes from young and old mice were stimulated for 19 h in culture. CHX was added 2 h before the end of culture. Results in Fig. 4 show that the addition of CHX to cultures significantly increases the stability of mRNA in T cells from old but not from young mice, the stability of T cells from the latter group already being very high. These findings suggest that the inhibition of protein translation renders the stability of mRNA in CD4\(^+\) lymphocytes from old mice identical to that of mRNA from young mice. Thus, the increased accumulation of IL-2 mRNA in CD4\(^+\) cells from young mice appears to be dependent on increased transcription rather than on increased stability of mRNA. These results together suggest that CHX treatment has blocked protein factors that influence mRNA transcription and stability.

**Evidence for a protein factor inhibiting IL-2 gene transcription**

To investigate whether the CHX-increased level of IL-2 mRNA may result from a decreased level of an IL-2-inhibiting protein, we have prepared nuclear extracts from activated CD4\(^+\) lymphocytes from young and old mice, and looked for the DNA binding activity of Nil-2-a, an inhibitor of the IL-2 gene (15) by an EMSA. Results in Fig. 5 show that nuclear extracts from unstimulated CD4\(^+\) cells from young mice exhibit higher levels of Nil-2-a as compared to CD4\(^+\) cells from old mice, whereas activated CD4\(^+\) cells (19 h) from old mice show significantly higher amounts of Nil-2-a as compared to young mice. Results from a preliminary experiment (not shown) indicated that a 10-fold excess of the unlabeled oligonucleotide completely removes the band due to Nil-2-a in the presence of an aspecific competitor. Moreover, the level of this inhibitor of the IL-2 gene seems to decrease in young but not in old mice with increasing time of stimulation. The effects of CHX on T cells from young mice were evident at both 3 and 19 h of stimulation. In a subsequent experiment (not shown), Nil-2-a was found decreased by 75% in young mice after 19 h of stimulation. Thus, in young mice, the decreased Nil-2-a level may account for the increased mRNA accumulation and expression with the time of stimulation. In old mice, the declined Nil-2-a level agrees with the slower increase in amount of mRNA and the reduced IL-2 production.

**Discussion**

The IL-2 gene is positively and negatively regulated, and different T cell subsets require different activation signals for IL-2 production (16). The cell concentration of IL-2 mRNA is dynamically regulated by its rate of synthesis and degradation. The main purpose of this study was to investigate the molecular mechanisms involved in IL-2 production in aging.

We have found age-related differences in IL-2 mRNA...
accumulation and expression consistent with the changes in the composition of the T CD4+ population in young and old mice. The peripheral pool of CD4+ naive T cells (CD44lowCD45RBhighMEL-14high3G11high), which has been shown to be predominant in young mice, decreases with age while that of memory T cells (CD44highCD45RBlowMEL-14low3G11low) increases (17).

The stability of IL-2 mRNA was found to be dependent upon the time of stimulation in T CD4+ cells from both young and old mice. However, the stability of IL-2 mRNA increases in T cells from young mice, whereas it declines in T cells from old mice with increasing time of stimulation. Our results parallel the finding that co-stimulation of CD4+ cells from young mice with anti-CD2 and anti-CD28 mAbs also progressively increases the stability of IL-2 mRNA during the time of activation (18). Our data on IL-2 mRNA stability in cells from young and old mice correlate by and large with those on IL-2 mRNA accumulation and expression. T CD4+ cells from old mice are more promptly activated as compared to cells from young mice in terms of the amount of mRNA and IL-2. These results are in agreement with the similar IL-2 mRNA stability in young and old mice after 16 h of stimulation. However, with the increasing time of activation, the cells from young mice bypass those from old mice, showing higher IL-2 production and IL-2 mRNA level. The difference in IL-2 concentration between culture supernatants, as seen after 16–40 h of stimulation, reflects very vigorous IL-2 production in young cells, even considering that IL-2 expression and IL-2 consumption is much lower in cells from old mice (2).

The increased stability of cytokine-specific mRNA in tumor cells as compared to normal cells suggests that neoplastic cells behave as maximally activated normal cells displaying high levels of stability and translation of mRNA (19). In line with this view, we have found that both the stability of IL-2 mRNA and the level of IL-2 protein increase with the time of stimulation of cells from young mice.

To investigate the mechanisms determining the different stability of IL-2 mRNA in young and old mice, in particular the role of proteins involved in stabilization, we performed experiments with CHX, known to inhibit the translation of labile proteins probably involved in IL-2 mRNA stabilization in cells from old mice, which are present in lower amounts at this time of stimulation in cells from young mice. Conversely, in the presence of CHX, CD4+ cells from young mice displayed superinduction of IL-2 mRNA but not increased stability. These data are consistent with the hypothesis of an increased transcription in CD4+ cells from young mice.

There is growing evidence for DNA binding factors able to control IL-2 gene transcription (16,20). The DNA control elements, indeed, seem to interact with either positive or negative factors depending on cell type and stimulation protocol. We have focused our interest on the mechanisms involved in negative control of IL-2 gene transcription.

Although CHX might have several unknown effects on activated T cells, our results are consistent with the hypothesis of a repressor factor which increases in activated T cells. This factor, Nil-2-a, is a zinc finger protein and is one of the negative regulatory factors for IL-2 (15). Nil-2-a has been described as containing proline/glutamine and aspartic/glutamic acid-rich sequences which function as transcriptional control domains in many proteins. The proximity of Nil-2-a binding sites to the elements which positively control IL-2 transcription, such as the binding sites for Oct-1 and AP-1, suggests that the effects of Nil-2-a may be mediated through its interference with the activity of these transcription factors. The transcriptional activity of Nil-2-a was demonstrated cotransfecting a human cell line with Nil-2-a expression vector and a plasmid containing IL-2 promoter sequence. Overexpression of Nil-2-a was found to inhibit pIL-2 LUC activity up to 70%. To explain the superinduction of IL-2 gene transcription in young but not in old mice after CHX treatment, we have investigated the presence of this inhibiting factor in resting and activated cells from young and old mice. We have found that only in young mice is there an effect of CHX on the Nil-2-a level. Preliminary data (not shown) from experiments performed using an anti-sense oligonucleotide to Nil-2-a, in order to block Nil-2-a mRNA translation, have shown a 40% increase in the IL-2-driven proliferation as compared to normal controls (untreated or sense-treated cultures). Our results may explain, at least in part, the up-regulation of IL-2 gene transcription in young mice. Nevertheless, we agree with the hypothesis that IL-2 gene transcription is primarily controlled by specific signaling pathways acting through proximal elements, while cis-elements exert secondary modulating effects. Future work should be performed to clarify the role of this inhibitory factor in IL-2-driven control of T cell proliferation.

Abbreviations

CHX cycloheximide
EMSA electrophoretic mobility shift assay
NRE negative regulatory element
WB washing buffer

References

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