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Serum amino acids, biopterin and neopterin during long-term immunotherapy with interferon-alpha in high-risk melanoma patients

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Abstract

Immunotherapy with interferon-alpha (IFN-α) induces neuropsychiatric side effects, most notably depression. One of the presumed pathophysiological mechanisms is an effect on tryptophan metabolism. As tryptophan is the precursor of serotonin, decreased availability of tryptophan to the central nervous system could result in serotonin deficiency. Tetrahydrobiopterin ($BH_{(4)}$) is a cofactor for one of the enzymes synthesizing serotonin. We conducted an exploratory study into the serum concentrations of large neutral amino acids (AA), biopterin (BIOP) and neopterin (NEOP), of 67 patients with high-risk melanoma, who were either treated with two different doses of IFN-α or were part of an observation-only control group. We found evidence for IFN-α to decrease concentrations of all AA except phenylalanine. The decrease in tryptophan concentration was most prominent and consistent. These changes persisted throughout a year of maintenance treatment. Concentrations of NEOP rose sharply, whereas, those of BIOP did not change. Except for the increase in NEOP and the increase in the ratio between phenylalanine (PHE) and tyrosine (TYR), no support for derangement in $BH_{(4)}$ metabolism was found. The increase in the ratio between PHE and TYR suggests inhibition of the enzyme phenylalanine hydroxylase. Patients with IFN-α induced anxiety and depression had higher pretreatment concentrations of NEOP. Changes in tryptophan metabolism may play a role in the pathophysiology of the neuropsychiatric side effects of IFN-α, and further research into the predictive potential of NEOP is warranted.

Keywords: Amino acids; Tryptophan; Interferon-alpha; Depression; Neopterin; Biopterin; Melanoma

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1. Introduction

Immunotherapy with interferon-alpha (IFN-α) induces neuropsychiatric side effects in high frequencies. Most notably, depressive disorders appear to be induced by IFN-α (Dusheiko, 1997; Dieperink et al., 2000). The pathophysiology underlying the induction of depressive disorders is probably related to the activation of the cytokine network and changes in serotonergic neurotransmission (Bonaccorso et al., 2001; Menkes and MacDonald, 2000). There is converging evidence that depression is accompanied by immunological derangement and even that immunological factors play a significant role in the pathophysiology of depression (Maes, 1995, 1997, 1999).

One of the putative mechanisms through which immune changes could influence mood and even induce depression is lowering of serum L-tryptophan (L-TRP), one of the essential amino acids (AA), with respect to the other large neutral AA (LNAA) (Capuron et al., 2002). The other LNAA [tyrosine (TYR), phenylalanine (PHE), isoleucine, leucine and valine] compete with L-TRP at the blood–brain barrier; a decrease of the L-TRP/LNAA ratio would set L-TRP at a relative disadvantage and cause a decreased availability of L-TRP to the central nervous system. As L-TRP is the precursor of serotonin and the availability of L-TRP is the rate-limiting step in serotonin synthesis by nerve cells, this in turn would lead to lower serotonin levels in the brain (Fernström and Wurtman, 1972).

Derangements in serotonergic functions are thought to play a central role in depression. Low L-TRP and low TRP/LNAA ratios in depressed patients have been reported (Maes et al., 1996). Treatment with cytokines is known to influence the metabolism of TRP, resulting in a decrease in L-TRP concentration and an increase in kynurenine excretion (Brown et al., 1989, 1991). Interferons stimulate the activity of tryptophan dioxygenase and indoleamine 2,3-dioxygenase (IDO), two enzymes degrading L-TRP, resulting in lowering of serum L-TRP levels (Brown et al., 1991; Taylor and Feng, 1991; Menkes and MacDonald, 2000). Another explanation for the low L-TRP levels might be that, as part of the immune response, L-TRP is preferentially used for proliferation of immune cells and the synthesis of acute phase proteins by the liver (Maes, 1997), or that decreased albumin levels as part of the immune response play a role (Maes et al., 1996).

Tetrahydrobiopterin ($BH_{(4)}$) is a necessary cofactor for various enzyme activities, among others, for the enzymes that hydroxylate L-TRP, TYR and PHE, which are the rate-limiting steps in the biosynthesis of serotonin, dopamine and noradrenaline, respectively (Thony et al., 2000). Therefore, a functional deficiency of $BH_{(4)}$ could result in a neurotransmitter deficiency. Deranged $BH_{(4)}$ metabolism has been implicated in the pathogenesis of mood disorders (Hashimoto et al., 1990, 1994; Hoekstra et al., 2001). One of the enzymes involved in the synthesis of $BH_{(4)}$, GTP-cyclohydrolase I, could be under control of cytokines (Thony et al., 2000; Van Amsterdam and Opperhuizen, 1999). Disturbance in $BH_{(4)}$ metabolism could be another pathophysiological mechanism in cytokine-induced depression. Neopterin (NEOP) concentrations are reported to increase during interferon treatment (Fuchs et al., 1992; Liberati et al., 1994). As NEOP is synthesized from the precursors of $BH_{(4)}$, treatment with IFN-α can possibly induce a deficiency of $BH_{(4)}$. The ratio of PHE to TYR (PHE/TYR-ratio) is an index for the activity of the enzyme synthesizing TYR from PHE (phenylalanine hydroxylase), which utilises $BH_{(4)}$ as a cofactor, so a raised PHE/TYR-ratio is suggestive of a $BH_{(4)}$ deficiency (Hoekstra et al., 2001).

The majority of the studies performed so far on the influence of treatment with cytokines on the metabolism of tryptophan and pteridines were in vitro studies. Studies in patients were mainly focused on short-term effects (Brown et al., 1991). We conducted an exploratory study into the serum concentrations of AA and biopterin (BIOP) and NEOP of patients with high-risk melanoma, who were either treated with IFN-α in two different doses or were part of an observation-only control group during a period of 1 year.

2. Materials and methods

The European Organization for Research and Treatment of Cancer (EORTC) performed a mul-

ticenter, randomized trial (EORTC 18952) evaluating the efficacy and toxicity of two total-dose-equivalent schedules of IFN-α-2b in high-risk melanoma patients. Patients underwent resection of a thick primary melanoma or regional lymph node metastases, in the absence of distant metastases. These patients have an over 50% chance of recurrence of their disease within 2 years. Patients were randomized in a 2:2:1 ratio for treatment arms A (1 year, high-intermediate dose), B (2 years, low-intermediate dose) or C (observation only). Our center enrolled 93 patients in the study. Patients in both arms A and B followed an induction phase with IFN-α for 4 weeks in a dose of 10 MIU s.c./day for 5 days a week; thereafter patients in arm A received 10 MIU three times a week s.c. for 1 year (total dose 1760 MIU) and patients in arm B received 5 MIU three times a week during 2 years (total dose 1760 MIU). Patients with organic mental disorders and with psychiatric disorders at baseline that could be exacerbated by IFN-α (e.g. depression) were not eligible.

Blood samples were taken at baseline, at the end of the 4-week induction phase, at 6 and 12 months. For practical reasons, it was not possible to obtain blood samples at fixed times or under fasting conditions. Case record forms and charts were reviewed separately on the eligibility and exclusion criteria. Samples were considered eligible when patients were using the planned IFN-α dose at the time of blood sampling. Samples were considered not to be eligible when patients were not using IFN-α at the desired dose level, the interferon was stopped or when possibly interfering comedication was used (e.g. antidepressants). As IFN-α was withdrawn once distant metastases occurred, no samples were analyzed from patients with distant metastatic disease.

3. Procedures

Blood was obtained by venipuncture and, after immediate centrifugation (10 min at $1000\times g$), serum was separated and frozen at −80°. All assays were performed within a period of 1–3 years after blood sampling. AA were determined as previously described (Fekkes et al., 1995). The L-TRP/LNAA-ratio was calculated by dividing 100 times the plasma concentration of L-TRP by the sum of the other LNAA, e.g. TYR, PHE, valine, leucine and isoleucine. The TYR/LNAA ratio was calculated in the same manner, substituting L-TRP for TYR and vice versa.

NEOP and BIOP were measured after acid oxidation of the reduced forms of both pteridines as described earlier (Fukushima and Nixon, 1980). Plasma (0.4 ml) was oxidized in 0.1 ml 1 M trichloroacetic acid and 0.05 ml iodine solution (0.5% I_2, 1% KI in 0.2 M trichloroacetic acid). For each assay, different amounts of NEOP and BIOP were added to a plasma pool in order to determine the percent recovery. After standing for 60 min under reduced light, excess iodine was reduced by the addition of 20 mol of 1% ascorbic acid solution and the mixture was centrifuged at $12\,000\times g$ for 15 min at 4 °C. The supernatant (0.4 ml) was transferred to an amber glass vial and 10 μl was injected directly onto the analytical column using an HPLC system with an autosampler and a fluorescence detector (Hewlett Packard, Series 1100). We used a Hypersil C_{18} column (2.1×200 mm, 5 μm), which was protected by a narrow bore guard column (2.1×20 mm) of the same material (Hewlett Packard). The separation was achieved using an aqueous 15 mmol/l potassium phosphate buffer, pH 6.45, and a stepwise eluent gradient with methanol (from 1.5 to 2 min to 10%, from 4 to 5 min to 100% and from 6 to 8.3 min to 0%). Total run time was 14 min, the flow rate was set at 0.4 ml/min, the column temperature was 50 °C, and the excitation and emission wavelengths were 360 and 440 nm, respectively. Compounds were quantitated by their peak height in comparison with external standards. NEOP and BIOP were eluted at approximately 2.2 and 4.8 min and the minimal signal distinguishable from baseline noise corresponded to an amount of both compounds of 2 and 3 fmol (0.2 and 0.3 nmol/l), respectively. Recoveries of NEOP and BIOP added to plasma samples were 100–140 and 95–110%, respectively. The intra-assay coefficients of variation for NEOP and BIOP were less than or equal to 2.4 and 2.1%, respectively. The interassay coefficients of variation for these com-

pounds, determined on 6 different days, were 3.8 and 2.8%, respectively.

Data were stored using SPPS software and analyzed using SAS software. Estimates of changes of parameters at follow-up compared to baseline were obtained using mixed model analysis of variance (mixed model ANOVA) after log transformation of the outcome variables. Effects of group (3 levels), time (3 levels) and their interaction on the change from baseline of the outcome variables were tested. Adjustment was made for the baseline measurement of the outcome variable at hand, sex, age and storage period by including them as covariates in the model. No structure was imposed on the correlation of the residuals. Differences between groups were assessed with the non-parametric Mann–Whitney *U*-test. Statistical significance was defined as $P<0.05$ (two-tailed).

4. Results

This study reports on the first 72 consecutive patients enrolled in the study in our center. Five subjects had to be excluded, mainly because of psychotropic drug use. Of the remaining 67 patients (34 males, 33 females, mean age 46.3 year, range 21–73), 28 belonged to treatment arm A, 29 to treatment arm B, and 10 to the observation group (C). There were samples missing due to administrative failure and, moreover, the number of available samples diminished in the course of time due to attrition (because of recurrent disease, treatment cessation or dose reduction in response to side effects, etc.)

In Table 1, baseline values and estimates (according to mixed model ANOVA) of percentual change compared to baseline are shown. During the 4-week induction phase (50 MIU/week), in the IFN-α treated groups, concentrations of L-TRP and the L-TRP/LNAA ratio showed a strongly significant decrease. Concentrations of TYR decreased but not the TYR/LNAA-ratio. In the induction phase of the IFN-α treated groups, the concentration significantly decreased for VAL by 13.1% (95% CI: −19.1/−6.7), for ILE by 22.6% (95% CI: −30.5/−13.8), and for LEU by 15.7% (95% CI: −24.8/−6.8) (data not shown). The concentration of PHE, however, did not change (data not shown). Interestingly, the PHE/TYR ratio (PHE/TYR ratio is a measure of the activity of phenylalanine hydroxylase, an enzyme that uses $BH_{(4)}$ as a cofactor) increased significantly. NEOP concentrations rose sharply, while BIOP concentrations did not change.

During maintenance treatment (with IFN-α in doses of 30 or 15 MIU/week), concentrations of L-TRP continued to be decreased, but the L-TRP/LNAA ratio was not significantly lowered except for the high-dose group at 6 months. Concentrations of TYR and the TYR/LNAA ratio did not differ from baseline during maintenance treatment. Concentrations of VAL and ILE at 6 months and concentrations of VAL, ILE and LEU at 12 months were decreased significantly compared to baseline (data not shown). The concentration of PHE was increased at 6 months (data not shown), and the PHE/TYR ratio was increased significantly at 6 and 12 months. Again, NEOP concentrations were elevated and BIOP concentrations did not diminish. No significant group by time interactions were found, and no significant differences between groups A and B emerged.

In the control group of 10 patients, changes in the opposite direction were seen. Compared to baseline, the concentration of VAL was raised at all points in time (data not shown), concentrations of L-TRP, TYR, PHE and LEU were raised at 6 and 12 months, and the concentration of ILE was raised at 6 months only (data on VAL, PHE, LEU and ILE not shown). The NEOP concentration did not change in the control group, while the BIOP level increased.

Ten patients (5 males, 5 females, mean age 50.4 years) were seen in psychiatric referral by the first author and clinically diagnosed with an IFN-α induced psychiatric disturbance. In eight patients, the psychiatric disturbance appeared during the high-dose induction phase; in two patients, during the maintenance period. Six out of ten patients had a depressive syndrome, in three of them rated as severe. One of these patients was admitted to a psychiatric hospital. Of the six depressed patients, one was successfully treated with an antidepressant and IFN-α could be continued; in two patients, IFN-α was stopped; two patients chose to continue IFN-α and to wait for the effects of dose reduction

Table 1
Baseline concentrations of AA, L-TRP/LNAA ratio, TYR/LNAA ratio, PHE/TYR ratio, BIOP and NEOP and estimations of percentual changes compared to baseline (adjusted for baseline value, sex, age and storage period; baseline = 100%)

	Treatment group	Mean concentration (S.D.) in 10^{-6} mol/l at baseline in groups A, B and C (59 samples)	Estimations of percentual changes (95% confidence interval) during follow-up compared to baseline (adjusted for baseline value, sex, age and storage period; baseline = 100%)		
			4 weeks (44 samples)	6 months (45 samples)	12 months (36 samples)
L-TRP	A	41.2 (7.1)	−24.1*** (−29.8/−18.0)	−15.3*** (−22.2/−7.8)	−17.1*** (−24.0/−9.6)
	B		−18.4*** (−24.0/−12.4)	−8.9* (−15.7/−1.6)	−10.8* (−17.9/−3.1)
	C		10.8 (−1.5/24.7)	23.7** (10.0/39.0)	21.2** (7.7/36.2)
TYR	A	65.6 (18.3)	−18.2** (−27.8/−7.4)	−6.1 (−17.1/6.5)	−12.5 (−24.8/0.5)
	B		−14.9* (−24.2/−4.5)	−2.3 (−12.7/9.4)	−9.0 (−20.1/3.7)
	C		17.8 (−2.1/41.8)	35.4* (13.9/60.9)	26.1* (5.2/51.3)
		Mean (S.D.)			
L-TRP/LNAA ratio	A	7.39 (1.34)	−11.2*** (−15.7/−6.4)	−9.1** (−14.2/−3.6)	−6.0 (−12.5/0.9)
	B		−5.8* (−10.2/−1.1)	−3.5 (−8.4/1.8)	−0.2 (−6.7/6.7)
	C		−7.1 (−14.5/1.1)	−4.8 (−12.1/3.2)	−1.6 (−10.0/7.6)
PHE/TYR ratio	A	1.00 (0.19)	28.0*** (17.6/39.4)	22.6*** (12.9/33.6)	22.1*** (11.1/34.1)
	B		16.5*** (7.4/26.3)	11.6** (3.5/20.3)	11.1* (1.5/21.5)
	C		−5.5 (−16.4/7.1)	−9.5 (−19.2/1.4)	−9.9 (−20.3/1.7)
		Mean concentration (S.D.) in 10^{-9} mol/l			
NEOP	A	7.7 (5.1)	185.9*** (134.2/249.0)	240.0*** (170.9/326.9)	266.8*** (188.4/366.4)
	B		131.8*** (94.3/175.9)	175.8*** (138.5/237.2)	197.4*** (138.5/271.0)
	C		−10.8 (−34.4/21.5)	6.2 (−22.3/45.0)	14.5 (−17.0/57.9)
BIOP	A	6.4 (2.0)	0.5 (−9.0/11.1)	2.5 (−8.8/13.8)	11.6* (0.1/24.4)
	B		−6.1 (−13.4/18)	−4.2 (−12.1/4.4)	4.2 (−5.1/14.3)
	C		26.9** (10.9/45.3)	29.5*** (13.3/47.9)	40.8*** (23.2/61.0)

Group A: 4 weeks 50 MIU/week, 48 weeks 30 MIU/week (28 patients); Group B: 4 weeks 50 MIU/week, 48 weeks 15 MIU/week (29 patients); Group C: observation only (10 patients). *$0.005 < P < 0.05$; **$0.0005 < P < 0.005$; ***$P < 0.0005$. These P-values relate to testing within-group changes from baseline.

after 4 weeks, and one patient decided to complete the remaining month of the maintenance phase. Four out of ten patients were clinically diagnosed with an IFN-α induced anxiety disorder and/or irritability, all during the induction phase. The complaints resolved after dose reduction according to the research protocol.

From the group seen in psychiatric referral and diagnosed with an IFN-α induced psychiatric disturbance, for seven patients, a baseline value was available. We compared the baseline values of these seven patients with the baseline values of the other patients of group A and B, as these patients were treated with interferon without being seen in psychiatric referral. NEOP concentrations at baseline in the group with an IFN-α induced psychiatric disturbance were significantly higher (mean 13.6, S.D. 11.7 vs. 6.9, S.D. 2.8, $P=0.002$) than in groups A and B. Other baseline values, most notably age, the L-TRP concentration and the L-TRP/LNAA-ratio, did not differ significantly.

5. Discussion

In this exploratory study, we show that IFN-α-based immunotherapy in cancer patients influences AA concentrations, both in short- and long-term measurements. Most notably, L-TRP concentrations decrease consistently. Concentrations of several other AA also decrease, most notably those of VAL and ILE. These changes are apparent during the induction phase with high-dose IFN-α, and persist to a lesser degree during a 1-year follow-up on a lower dose. Concentrations of PHE do not change or even increase. The ratio of L-TRP to the other LNAA, competing for the same active transport mechanism, decreases during the induction phase and is still lower at 6 months in the high-dose group.

The decrease in L-TRP concentrations is in accordance with earlier work (Brown et al., 1991) and probably reflects a permanent induction of IDO, one of the enzymes catabolising L-TRP (Brown et al., 1991; Taylor and Feng, 1991; Menkes and MacDonald, 2000). Furthermore, one can speculate that dietary changes play a role. Recently, Bonaccorso et al. showed that, in hepatitis patients, IFN-α based immunotherapy over 24 weeks decreased concentrations of plasma L-TRP and of the competing AA, decreased serotonin levels in serum, and increased kynurenine (the degradation product of L-TRP) concentrations (Bonaccorso et al., 2002). Capuron et al. found, in cancer patients on IFN-α treatment, a decrease of L-TRP and of the L-TRP/LNAA ratio, during a period of 1 month (Capuron et al., 2002).

L-TRP is the precursor of serotonin. A decrease of the L-TRP/LNAA ratio would set L-TRP at a relative disadvantage in competing with other LNAA at the blood–brain barrier and cause a decreased availability of L-TRP to the central nervous system. Bonaccorso et al. found a relationship between kynurenine concentration and the score on the Montgomery–Åsberg depression rating scale (Bonaccorso et al., 2002). Capuron et al. found a relationship between the decrease in L-TRP concentrations and the development and severity of several depressive symptoms, including pessimistic thoughts and suicidal ideation (Capuron et al., 2002). These observations support the hypothesis that reduced availability of L-TRP influences the serotonergic neurotransmission and is of pathophysiological relevance to the development of neuropsychiatric side effects of IFN-α.

As in earlier reports (Fuchs et al., 1992; Liberati et al., 1994), high-dose IFN-α also induces a sharp increase of NEOP, indicating activation of cellular immunity. Theoretically, the increased synthesis of NEOP could hamper the synthesis of $BH_{(4)}$ by shunting away the precursors of $BH_{(4)}$. This would be compatible with the consistently observed increase in the PHE/TYR ratio, indicative of a diminished synthesis of TYR from PHE. The enzyme synthesizing TYR from PHE (phenylalanine hydroxylase) utilises $BH_{(4)}$ as a cofactor, so a raised PHE/TYR-ratio is compatible with a $BH_{(4)}$ deficiency. However, total BIOP concentrations (the sum of $BH_{(4)}$ and its oxidative products, dihydrobiopterin and BIOP) do not change, which does not point to $BH_{(4)}$ deficiency. According to some investigators, measurements of BIOP concentrations reflect to a large extent the concentration of $BH_{(4)}$ (Hashimoto et al., 1990).

We found opposite changes in the control group, i.e. the concentrations of several AA including L-TRP and BIOP increase compared to baseline. The

NEOP concentrations and the PHE/TYR ratio did not change. One should take into account that group C was smaller as a consequence of the randomisation rate (2:2:1). But as most patients had surgery in the weeks preceding baseline, one could speculate that the changes in group C reflect a recovery from postoperative catabolism. As concentrations of AA and most prominently L-TRP increase in group C and decrease in treated groups, this would imply that the size of the effect of treatment with IFN-α on AA concentrations is even greater. Our findings suggest that future research into the influence of immunotherapy on AA metabolism in cancer patients should include an untreated control group of sufficient size.

Patients seen in psychiatric referral had higher baseline concentrations of NEOP, suggestive of a pre-existing activation of cellular immunity in this group that could increase the vulnerability to development of neuropsychiatric side effects. Our finding is in line with the previously reported raised NEOP concentrations in depressed patients (Dunbar et al., 1992; Maes et al., 1993).

More research is needed to relate changes in AA and $BH_{(4)}$ metabolism to behavioral change and to the development of psychiatric disturbance. This could demonstrate the relevance of laboratory changes to the pathophysiology of IFN-α induced neuropsychiatric side effects.

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