

Letter to the Editor

Advanced glycation end products contribute to the immunogenicity of IFN- β pharmaceuticals

To the Editor:

Human IFN- β proved to be efficient for the treatment of relapsing-remitting multiple sclerosis. There are 2 forms of recombinant human IFN- β on the market, IFN- β 1a (Avonex [Biogen Idec Inc, Weston, Mass] and Rebif [Merck Serono S.A., Geneva, Switzerland]) and IFN- β 1b (Betaferon and Betaseron [Bayer HealthCare Pharmaceuticals Inc, Berlin, Germany]). IFN- β 1a is produced in Chinese hamster ovary cells, whereas IFN- β 1b is derived from *Escherichia coli*. The latter is a nonglycosylated form with a C17S substitution. Formation of neutralizing antibodies against human IFN- β has been reported for all IFN- β therapeutics, with the percentage of neutralizing antibody-positive patients varying between 3% (Avonex) and 35% (Betaferon).¹ The most frequent side effects observed with IFN- β are injection-site reactions and flu-like symptoms. Cases of type 1 hypersensitivity reactions, including urticaria² and severe anaphylaxis,³ have also been reported.

The factors causing the development of antibodies against human IFN- β remain elusive. We have shown previously that *E coli*-derived human IFN- γ ⁴ and consensus human IFN- α ⁵ are modified with advanced glycation end products (AGEs). AGEs represent a heterogeneous group of carbohydrate adducts formed in the Maillard reaction, which is also known as glycation. Protein-derived AGEs are formed in both eukaryotic and prokaryotic cells. Therefore we hypothesized that human IFN- β produced in either Chinese hamster ovary cells (IFN- β 1a) or *E coli* (IFN- β 1b) will accumulate AGEs. Some protein-bound AGEs are bulky carbohydrate derivatives, which might act like haptens, rendering self-proteins immunogenic. In fact, experimental data support involvement of both innate⁶ and adaptive⁷ immune reactions against AGEs. This study was designed to test the hypothesis that AGEs form immunogenic epitopes on IFN- β .

We first tested the IFN- β pharmaceuticals Avonex, Rebif, and Betaferon for the presence of 2 AGEs, N^ε-(carboxymethyl)lysine (CML) and imidazolone, by means of competitive ELISA (for more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org). All drugs proved to contain CML and imidazolone, with Rebif exhibiting the highest levels

of both AGE modifications. CML (means $\pm$ CI, $n = 6$) as expressed in milligram-equivalent (mg-eq) AGE-modified BSA (AGE-BSA) per milligram of IFN- β was 1.3 ± 0.1 mg-eq (Avonex), 12.1 ± 1.4 mg-eq (Rebif), and 1.0 ± 0.1 mg-eq (Betaferon); imidazolone was 0.6 ± 0.1 mg-eq (Avonex), 5.1 ± 0.7 mg-eq (Rebif), and 0.6 ± 0.1 mg-eq (Betaferon). We measured a very low concentration of CML and imidazolone in pharmaceutical grade human serum albumin (HSA), 0.005 ± 0.001 and 0.001 ± 0.0006 mg-eq AGE-BSA per milligram of HSA, respectively, which implies that both AGE modifications in Avonex and Betaferon are derived mostly from IFN- β but not from the excipient HSA in these drugs. This conclusion found further support in the following experiment. Betaferon preparations were subjected to size exclusion (SE) HPLC to extract IFN- β 1b from the excess HSA. The IFN- β 1b-enriched fraction (no. 1) thus obtained contained about 15 times more CML than the HSA fraction (no. 2): 25.4 ± 3.4 versus 1.6 ± 0.4 μ g-eq AGE-BSA per milliliter fraction (no. 1 and no. 2, respectively). In the SE-HPLC experiment IFN- β 1b (37.0 kd) was preferably eluted in the first fraction to the exclusion of HSA (66.4 kd; [Fig 1, A](#)), suggesting aggregation of IFN- β 1b, which was confirmed also by means of native polyacrylamide gel electrophoresis (PAGE; [Fig 1, B](#)). Noncovalent aggregation of IFN- β 1b, which is generally attributed to the lack of native glycosylation, is well documented in the literature and known to result in reduced IFN- β 1b biologic activity. Covalent aggregation, however, accompanied by protein degradation is a typical feature of AGE-modified and oxidized proteins. To test for such changes, we performed SDS-PAGE under reducing conditions of the IFN- β 1b-enriched SE-HPLC fraction no. 1 followed by immunoblotting with an anti-IFN- β antibody and detected both truncated products and higher-order oligomers ([Fig 1, C](#)). Similar derivatives, which might further contribute to IFN- β immunogenicity, were observed also in IFN- β 1a ([Fig 1, D](#)).

To explore the immunogenic potential of AGEs, we studied sera from patients with multiple sclerosis treated with either IFN- β 1a (Avonex and Rebif groups) or IFN- β 1b (Betaferon group) for the presence of anti-IFN- β and anti-AGE IgG antibodies. Seventy-four percent (31/42) of the patients enrolled in this study had flu-like symptoms, injection-site reactions, or both (for patients' clinical characteristics, see [Tables E1-E3](#) in this article's Online Repository at www.jacionline.org). Informed consent was

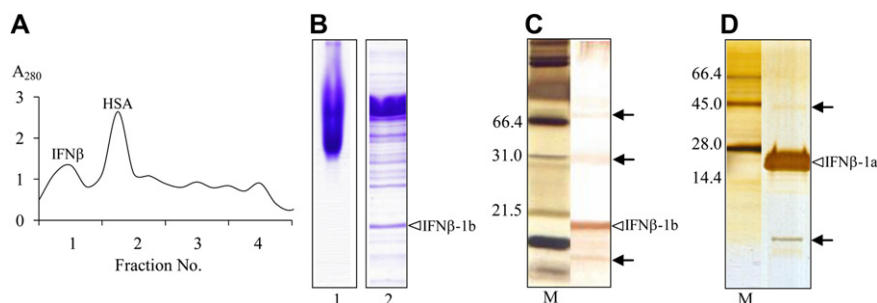


FIG 1. IFN- β 1a and IFN- β 1b have undergone structural alterations. **A**, SE-HPLC chromatogram of Betaferon. **B**, Native PAGE (lane 1) and SDS-PAGE (lane 2) of Betaferon. **C**, Immunoblot of Betaferon SE-HPLC fraction no. 1 with an anti-IFN- β antibody. **D**, Silver-stained SDS polyacrylamide gel of Rebif. In [Fig 1, C and D](#), degradation and covalent aggregation are indicated by solid arrows, and *M* stands for molecular mass standards in kilodaltons.

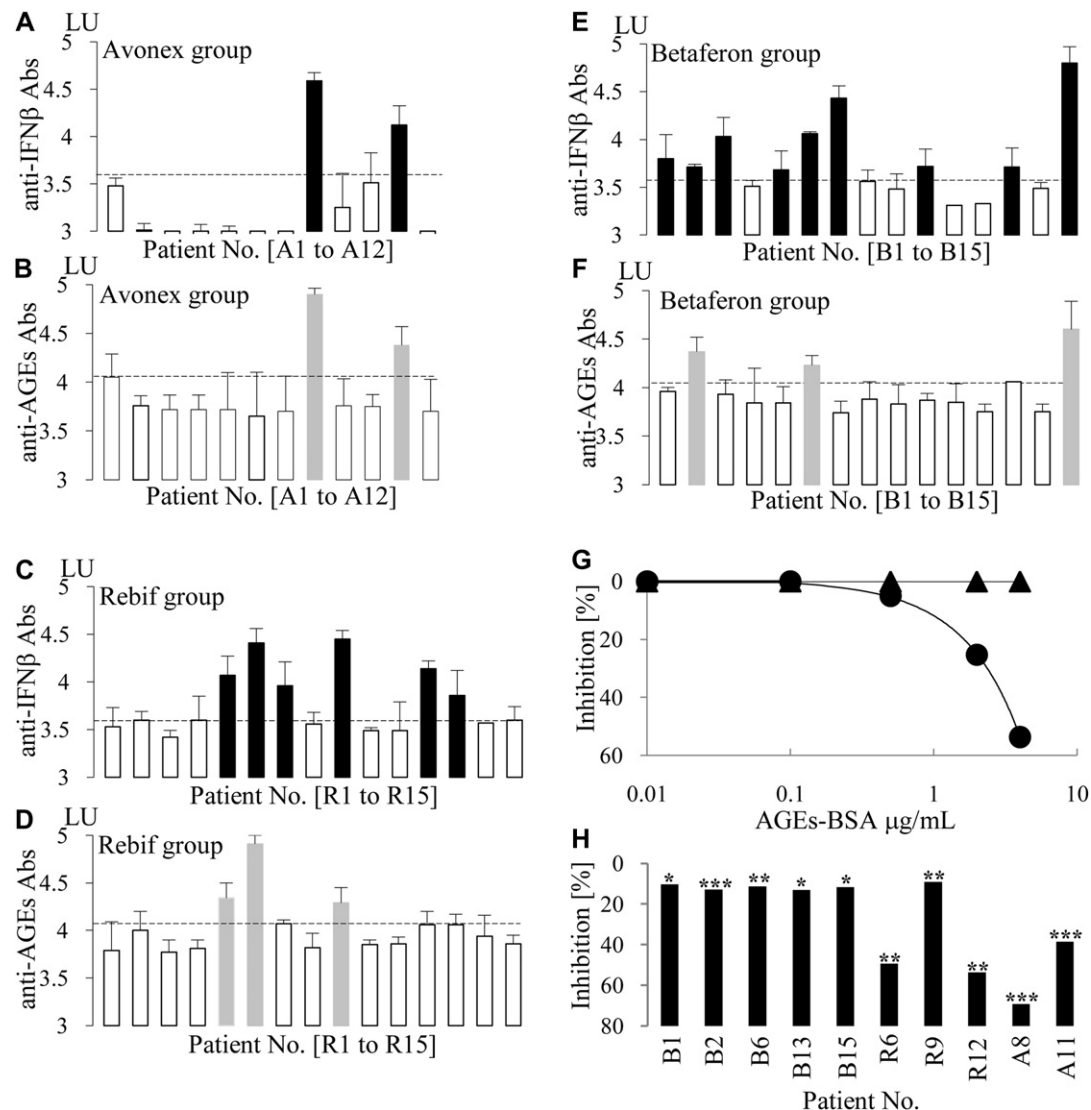


FIG 2. Titers of anti-IFN- β (**A**, **C**, and **E**) and anti-AGE (**B**, **D**, and **F**) antibodies. Data are means $\pm$ SDs ($n = 2$) and are representative of 3 independent experiments. Sera from patients with multiple sclerosis with average titers greater than the mean $+ 3$ SDs of the control group (dashed line) were considered positive (black and gray bars). **G**, Inhibition of the reactivity of sera R6 (solid circles) and B7 (solid triangles) to IFN- β by AGE-BSA. **H**, Anti-IFN- β antibody-positive sera in which the reactivity to IFN- β was significantly ($P < .05$) inhibited by AGE-BSA. In Fig 2, **G** and **H**, inhibition is presented as the percentage difference in sera reactivity to IFN- β in the presence of either AGE-free (100%) or AGE-modified BSA. Data are means from 8 replicates, and the difference was tested for statistical significance by using the paired 2-tailed Student t test. * $P < .05$, ** $P < .001$, and *** $P < .0001$.

obtained from all patients before examination of their sera. Seventeen percent (2/12) of the sera from the Avonex group, 40% (6/15) of the sera from the Rebif group, and 60% (9/15) of the sera from the Betaferon group were found to be anti-IFN- β antibody positive (Fig 2, **A**, **C**, and **E**). Seventeen percent (2/12) of the sera in the Avonex group and 20% of the sera in the Rebif and Betaferon groups (3/15 and 3/15) contained anti-AGE antibodies also (Fig 2, **B**, **D**, and **F**). The serum levels of anti-IFN- β antibodies correlated positively with the levels of anti-AGE antibodies, especially in the Avonex and Rebif groups, with Pearson correlation coefficients (r) of 0.95 ($P < .001$) and 0.89 ($P < .001$), respectively. The correlation coefficient for the Betaferon group was an r value of 0.65 ($P < .009$; for more

information, see Fig E1 in this article's Online Repository at www.jacionline.org). The serum of patient B15 was analyzed for the titer of anti-IFN- β and anti-AGE antibodies 2 years after the initial testing while IFN- β was still being administered. In this patient we observed the same directional dynamics of both types of antibodies represented by a 35.9% decrease ($P < .001$) in the titer of the anti-IFN- β antibodies and a 22.8% decrease ($P < .001$) in the titer of the anti-AGE antibodies.

To further explore the immunogenic potential of AGEs, we tested the serum anti-IFN- β antibodies for cross-reactivity with AGEs. We included AGE-BSA as a competitor in the reaction between serum IgGs and IFN- β . Initially, we performed competitive ELISA with 2 sera, one that was anti-IFN- β antibody

positive and anti-AGE antibody negative (B7) and another that was positive for both types of antibodies (R6). Increasing concentrations of AGE-BSA progressively inhibited the reactivity to IFN- β of the anti-AGE antibody-positive serum (R6) but not the reactivity of the anti-AGE antibody-negative serum (B7; Fig 2, G). All anti-IFN- β antibody-positive sera were tested in a similar manner at a fixed concentration of the competitor. The 2 anti-IFN- β antibody-positive sera in the Avonex group (2/12), more than half of the anti-IFN- β antibody-positive sera in the Betaferon group (5/9), and half of the anti-IFN- β antibody-positive sera in the Rebif group (3/6) responded to AGE-BSA inhibition with a response range between 9.2% (R9) and 69.3% (A8; Fig 2, H), reflecting the relative contribution of AGEs to the overall IFN- β immunogenicity in each patient. The response of the anti-AGE antibody-negative sera R12, B1, and B13 to inhibition by AGE-BSA suggests that these sera were most likely determined to have false-negative results in the primary screening. As seen in Fig 2, D and F, their titers decrease near the cutoff value between negative and positive sera. All 10 sera responding to inhibition by AGE-BSA were further tested for response to mAbs raised against CML and imidazolone. Significant inhibition ($P < .05$) of sera reactivity to IFN- β was observed with sera A8, R6, and R12. In each of these sera, inhibition by the anti-CML antibody was stronger (20.5%, 15.9%, and 13.6% for A8, R6, and R12, respectively) compared with the inhibition caused by the anti-imidazolone antibody (14.3%, 7.2%, and 5.9% for A8, R6, and R12, respectively).

The cross-reactivity of the anti-IFN- β antibodies with AGEs implies that AGEs form immunogenic epitopes on IFN- β . A role for the basic subunit of the peanut Ara h 3 protein, which is prone to AGE modification, in the allergenicity of peanuts has been proposed.⁸ In the last decade, it has been convincingly demonstrated that AGEs are recognized by a receptor for advanced glycation end products (RAGE) belonging to the immunoglobulin superfamily. This receptor is expressed on a number of cells, including inflammatory cells, such as monocytes. The AGE CML, which we detected in IFN- β , has been reported to interact with RAGEs and to activate proinflammatory pathways.⁹ Taken together, the formation of anti-AGE-IFN- β antibodies and RAGE-promoted inflammation might contribute to reduced treatment efficacy and to some of the side effects seen in patients on medication with IFN- β .

In conclusion, to the best of our knowledge, this is the first study providing evidence for the presence of nonnative determinants of a well-defined origin (AGEs) in human IFN- β . Further

clinical and laboratory investigations are required to evaluate the effect of AGEs on the therapeutic and safety properties of IFN- β . In addition, appropriate strategies have to be adopted by pharmaceutical companies for the manufacture of AGE-free IFN- β with reduced immunogenicity.

Angelina Bozhinov, PhD^a
Yordan Handzhiyski, PhD^a
Krasimir Genov, MD, PhD^b
Vera Daskalovska, MD, DSc^c
Toshimitsu Niwa, MD, PhD^d
Ivan Ivanov, DSc^a
Roumyana Mironova, PhD^a

From ^athe Department of Gene Regulations, Institute of Molecular Biology "Roumen Tsanev," Bulgarian Academy of Sciences, Sofia, Bulgaria; ^bthe Department of Neurology and Neurosurgery, Military Medical Academy, Sofia, Bulgaria; ^cthe Department of Neurology, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Macedonia; and ^dthe Department of Advanced Medicine for Uremia, Nagoya University Graduate School of Medicine, Nagoya, Japan. E-mail: rumym@bio21.bas.bg.

Disclosure of potential conflict of interest: R. Mironova has received research support from the National Science Fund of Bulgaria. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Malucchi S, Sala A, Gilli F, Bottero R, Di Sapio A, Capobianco M, et al. Neutralizing antibodies reduce the efficacy of betaIFN during treatment of multiple sclerosis. *Neurology* 2004;62:2031-7.
2. Gujjarro C, Benito-León J, Bermejo-Pareja F. Widespread urticaria due to intramuscular interferon beta-1a therapy for multiple sclerosis. *Neurol Sci* 2011;32:309-11.
3. Corona T, Leon C, Ostrosky-Zeichner L. Severe anaphylaxis with recombinant interferon beta. *Neurology* 1999;52:425.
4. Mironova R, Niwa T, Dimitrova R, Boyanova M, Ivanov I. Glycation and post-translational processing of human interferon-gamma expressed in *Escherichia coli*. *J Biol Chem* 2003;278:51068-74.
5. Mironova R, Sredovska A, Ivanov I, Niwa T. Maillard reaction products in the *Escherichia coli*-derived therapeutic protein interferon alfacon-1. *Ann N Y Acad Sci* 2008;1126:181-4.
6. Liliensiek B, Weigand MA, Bierhaus A, Nicklas W, Kasper M, Hofer S, et al. Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J Clin Invest* 2004;113:1641-50.
7. Damasiewicz-Bodzek A, Wielkoszyński T. Advanced protein glycation in psoriasis. *J Eur Acad Dermatol Venereol* 2011 [Epub ahead of print].
8. Guo B, Liang X, Chung SY, Maleki SJ. Proteomic screening points to the potential importance of Ara h 3 basic subunit in allergenicity of peanut. *Inflamm Allergy Drug Targets* 2008;7:163-6.
9. Humpert PM, Lukic IK, Thorpe SR, Hofer S, Awad EM, Andrassy M, et al. AGE-modified albumin containing infusion solutions boosts septicemia and inflammation in experimental peritonitis. *J Leukoc Biol* 2009;86:589-97.

doi:10.1016/j.jaci.2011.10.035

METHODS

Patients and pharmaceuticals

Patients with relapsing-remitting multiple sclerosis, 13 Bulgarians (Military Medical Academy, Sofia, Bulgaria) and 29 Macedonians (Clinical Center, Skopje, Macedonia), were treated for at least 6 months once weekly intramuscularly with Avonex 30 µg (Biogen Idec Ltd, Maidenhead, United Kingdom), 3 times weekly subcutaneously with Rebif 44 µg Original Formulation (Serono S.A., Bari, Italy), or every other day subcutaneously with Betaferon (Bayer-Schering Pharma AG, Berlin, Germany). The average age of the Avonex-treated (12 Macedonians), Rebif-treated (15 Macedonians), and Betaferon-treated (13 Bulgarians and 2 Macedonians) patients was 34.5, 39.8, and 41.2 years, respectively. Sera from 15 anonymous blood donors (Macedonians) served as controls. Rebif 44 µg New Formulation (Merck Serono, Bari, Italy) was used for *in vitro* analyses. All formulations were tested before the expiration date.

Direct ELISA for measurement of anti-IFN-β and anti-AGE antibodies

AGE-BSA was prepared by incubating 10 mg/mL BSA with 0.5 mol/L glucose for 3 months at 37°C. ELISA plates (Costar; Corning, Inc, Corning, NY) were coated with 100 µL per well of either 10 µg/mL AGE-BSA or 1.5 µg/mL IFN-β (Betaferon) diluted in 50 mmol/L sodium carbonate buffer (pH 9.6). Corresponding blank wells were coated with carbonate buffer only. After overnight incubation at 37°C, coating solution was decanted, and wells were blocked for 3 hours at 37°C with PBS containing 3% BSA. After 2 washing steps with PBS containing 0.05% Tween 20 and 1 washing with PBS, 100-µL serum samples diluted 1:500 in PBS containing 1% BSA were added in duplicates to the test and blank wells. Nine 2-fold serial dilutions of serum B15 starting at 32× were used to construct a standard curve, and plates were incubated for 2 hours at 37°C. Next, plates were washed 3 times as above and incubated for 1 hour at 37°C with 100 µL per well of horseradish peroxidase (HRP)-conjugated goat antibody to human IgG (IgG-HRP; BulBio-NCIPD Ltd, Sofia, Bulgaria) diluted 1:4000 in 0.25% BSA in PBS. After 3 washing steps, color was developed by adding 100 µL per well of 1 mg/mL *o*-phenylenediamine in 0.2 mol/L K₂HPO₄ citrate buffer (pH 6.0) containing 0.02% H₂O₂. The reaction was stopped with 100 µL per well of 0.8 mol/L H₂SO₄, and the absorption at 490 nm was read with a microplate reader (ELx 800 ELISA Reader; Bio-Tek Instruments, Inc, Winooski, Vt). Optical densities (OD₄₉₀) of the blank wells were subtracted from the OD₄₉₀ of the test wells, and titers of the standard serum B15 (1:64,000 for anti-IFN-β antibodies and 1:32,000 for anti-AGE antibodies) were defined as the highest dilution at which ΔOD₄₉₀ was still greater than 0.1. Antibody titers of serum samples were determined by using the standard curve and converted to laboratory units (LU) by calculating the log₁₀ of the titer reciprocals. Sera from patients with multiple sclerosis with average titers of greater than the mean + 3 SD of the control group were considered positive. The cutoff values for the anti-IFN-β and anti-AGE antibodies thus calculated were 3.6 LU (2.7 + 3 × 0.30) and 4.1 LU (3.1 + 3 × 0.33), respectively.

Competitive ELISA for measurement of cross-reactivity of anti-IFN-β antibodies with AGEs

ELISA plates were coated overnight at 37°C with 100 µL per well of 1.5 µg/mL IFN-β (Betaferon) in 50 mmol/L sodium carbonate buffer (pH 9.6). Then the coating solution was replaced with 3% BSA dissolved in PBS, and after blocking for 3 hours at 37°C, plates were washed twice with 0.05% Tween 20 in PBS and once with PBS. In test wells 8 replicates of 50 µL of anti-IFN-β antibody-positive sera diluted 8× with 1% BSA in PBS were mixed with 50 µL of AGE-BSA in PBS at a concentration of 20 µg/mL. Control wells were prepared in the same way, except that AGE-BSA was replaced with nonglycated BSA (AGE-free BSA). When competition was carried out with anti-AGE antibodies, sera in test wells were mixed with 50 µL of either anti-CML^{E1} or anti-imidazolone^{E2} mAbs diluted in 0.25% BSA in PBS at 1:400 and 1:2000, respectively. In this case heat-inactivated anti-CML and anti-imidazolone antibodies served as negative controls. A competition reaction was carried out at 37°C for 2 hours, and after 3 washing steps, 100 µL of goat anti-human IgG-HRP diluted 1:4000 in 0.25% BSA in PBS was added to each well. Plates were incubated for 1 hour

at 37°C, and after 3 washing steps, color was developed as described above for direct ELISA. Inhibition of sera reactivity to IFN-β by AGE-BSA and anti-AGE antibodies was expressed as the percentage difference in sera reactivity in control (100%) and test wells. The difference was tested for statistical significance by using the paired 2-tailed Student *t* test.

Competitive ELISA for measurement of CML and imidazolone

EILSA plates were coated overnight at 37°C with 100 µL per well of AGE-BSA solution at a final concentration of either 10 µg/mL (for CML quantitation) or 1 µg/mL (for imidazolone quantitation) in 50 mmol/L sodium carbonate buffer (pH 9.6). Plates were washed 2 times with 0.05% Tween 20 in PBS and once with PBS. AGE-BSA was diluted in PBS at concentrations ranging from 4 to 0.002 mg/mL and used as a standard. Betaferon and Avonex (powders) were reconstituted according to the manufacturer's instructions, whereas Rebif (liquid) and pharmaceutical grade 20% HSA solution (BulBio-NCIPD Ltd) were used directly. Fifty microliters of samples and standards in duplicate 2-fold serial dilutions in PBS was mixed with 50 µL of HRP-labeled anti-CML^{E1} or anti-imidazolone^{E1} antibody diluted 1:1000 in 0.25% BSA in PBS. A competition reaction was carried out for 3 hours at 37°C, and after 3 washing steps, color was developed as described for direct ELISA. Data were evaluated with a 4-parameter logistic fitting curve. The amount of CML and imidazolone was normalized to the interferon content in the drugs and expressed as mass equivalent AGE-BSA per milligram of IFN-β. Three vials of each drug were analyzed in duplicates, and the pooled results were presented as means ± CIs.

SE-HPLC of Betaferon

Betaferon was fractionated by means of SE-HPLC by using an LKB Bromma HPLC system equipped with an LKB/Ultrapac TSK-G3000SW column (7.5 × 300 mm) and PBS as a mobile phase at a flow rate of 0.3 mL/min. The CML concentration of SE-HPLC fractions no. 1 and no. 2 was measured by using competitive ELISA, as described above.

PAGE of Rebif and Betaferon

Rebif was concentrated by using the Biomax-5 membrane with a relative molecular mass cutoff of 5 kd (Millipore, Corp, Billerica, Mass), whereas Betaferon was reconstituted according to the manufacturer's recommendations. SDS-PAGE of Betaferon (50 µg of total protein) and Rebif (10 µg) was performed according to the method of Laemmli^{E3} under reducing conditions (0.7 mol/L 2-mercaptoethanol) by using 18% gels. In parallel, Betaferon (50 µg of total protein) was separated on 10% acidic native gel under nonreducing conditions. For preparation of both types of gels, a stock solution of acrylamide/N,N'-methylene-bis-acrylamide (30/0.4) was used. Native gel electrophoresis was performed in a discontinuous buffer system (125 mmol/L sodium acetate in the stacking gel, 375 mmol/L sodium acetate in the separating gel, and 140 mmol/L acetic acid/0.33 mol/L β-alanine in the electrode buffer, pH 4.3).^{E4} After separation of proteins at a constant current of 30 mA, gels were stained with either 0.025% Coomassie Brilliant Blue R250 (in 10% acetic acid and 25% methanol) for Betaferon or 0.8% AgNO₃ (in 40 mmol/L NaOH and 0.4% NH₄OH) for Rebif. The Coomassie-stained gel was destained in 10% acetic acid and 25% methanol, whereas the silver staining reaction was stopped by placing the gel in a solution of 5% acetic acid.

Western blotting of Betaferon

The IFN-β1b-enriched SE-HPLC fraction no. 1 (Betaferon) was concentrated by means of ultrafiltration through a Biomax-5 membrane with a relative molecular mass cutoff of 5 kd, and 50 µg of total protein was separated on 18% SDS-polyacrylamide gels, as described above. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes for 40 minutes at a constant current of 200 mA in a blotting buffer consisting of 0.25 mol/L Tris base, 0.19 mol/L glycine, and 5% methanol. The membrane was blocked with 2% BSA in PBS overnight at 4°C and then treated with a

rabbit anti-human IFN- β IgG (Sigma-Aldrich Corp, St Louis, Mo), which was added directly to the blocking solution at a titer of 1:10,000. After incubation for 2 hours at room temperature (RT) while shaking, the membrane was washed 2 times with 0.05% Tween 20 in PBS and once with PBS. Each washing step was carried out for 20 minutes while shaking at RT. Next, the membrane was incubated with a goat anti-rabbit IgG-HPR (Biotrend Chemikalien GmbH, Cologne Germany) diluted 1:50,000 in 2% BSA in PBS for 1 hour at RT. After 3 washing steps, as above, the membrane was stained with 0.08% diaminobenzidine dissolved in 25 mmol/L Tris-HCl (pH 7.5) and supplemented with 0.03% hydrogen peroxide.

REFERENCES

- E1. Niwa T, Sato M, Katsuzaki T, Tomoo T, Miyazaki T, Tatemichi N, et al. Amyloid b2-microglobulin is modified with N1-(carboxymethyl) lysine in dialysis-related amyloidosis. *Kidney Int* 1996;50:1303-9.
- E2. Niwa T, Katsuzaki T, Miyazaki S, Momoi T, Akiba T, Miyazaki T, et al. Amyloid b2-microglobulin is modified with imidazolone, a novel advanced glycation end product, in dialysis-related amyloidosis. *Kidney Int* 1997;51:187-94.
- E3. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- E4. Reisfeld RA, Lewis UJ, Williams DE. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* 1962;195:281-3.

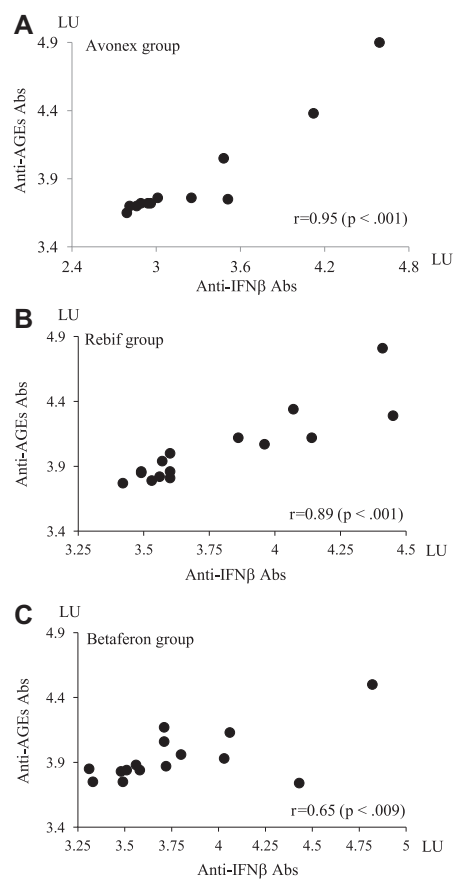


FIG E1. Correlation of the levels of anti-IFN- β antibodies with the levels of anti-AGE antibodies in the sera of patients with multiple sclerosis treated with Avonex (**A**), Rebif (**B**), and Betaferon (**C**). Correlation coefficients were calculated with the CORREL function of Microsoft Office Excel 2003 (Microsoft, Redmond, Wash), and their statistical significance was evaluated (<http://faculty.vassar.edu/lowry/rsig.html>).

TABLE E1. Clinical characteristics of patients with multiple sclerosis on medication with Avonex

Patient no.	Anti-IFN- β antibodies (LU)	Anti-AGE antibodies (LU)	Inhibition by AGE-BSA (%)*	Flu-like symptoms	Injection-site reactions	Disability progression
A1	Negative	Negative	NT	No	No	No
A2	Negative	Negative	NT	Yes	No	No
A3	Negative	Negative	NT	No	No	No
A4	Negative	Negative	NT	Yes	No	No
A5	Negative	Negative	NT	No	No	No
A6	Negative	Negative	NT	Yes	No	No
A7	Negative	Negative	NT	No	No	No
A8	4.59	4.90	69.3	Yes	Yes	No
A9	Negative	Negative	NT	Yes	No	No
A10	Negative	Negative	NT	No	No	Yes
A11	4.12	4.38	38.6	Yes	No	Yes
A12	Negative	Negative	NT	No	No	No

NT, Not tested.

*Inhibition of sera reactivity to IFN- β by AGE-BSA (for details, see [Fig 2](#), *G* and *H*).

TABLE E2. Clinical characteristics of patients with multiple sclerosis on medication with Rebif

Patient no.	Anti-IFN- β antibodies (LU)	Anti-AGE antibodies (LU)	Inhibition by AGE-BSA (%)*	Flu-like symptoms	Injection-site reactions	Disability progression
R1	Negative	Negative	NT	Yes	No	Yes
R2	Negative	Negative	NT	Yes	Yes	No
R3	Negative	Negative	NT	No	No	No
R4	Negative	Negative	NT	Yes	No	No
R5	4.07	4.34	0.0	Yes	Yes	Yes
R6	4.41	4.91	49.4	Yes	Yes	Yes
R7	3.96	Negative	0.0	Yes	Yes	Yes
R8	Negative	Negative	NT	Yes	No	Yes
R9	4.45	4.29	9.2	Yes	No	Yes
R10	Negative	Negative	NT	Yes	Yes	No
R11	Negative	Negative	NT	Yes	No	Yes
R12	4.14	Negative	53.7	Yes	Yes	Yes
R13	3.86	Negative	0.0	Yes	Yes	No
R14	Negative	Negative	NT	Yes	No	No
R15	Negative	Negative	NT	Yes	No	No

NT, Not tested.

*Inhibition of sera reactivity to IFN- β by AGE-BSA (for details, see [Fig 2](#), *G* and *H*).

TABLE E3. Clinical characteristics of patients with multiple sclerosis on medication with Betaferon

Patient no.	Anti-IFN- β antibodies (LU)	Anti-AGE antibodies (LU)	Inhibition by AGE-BSA (%)*	Flu-like symptoms	Injection-site reactions	Disability progression
B1	3.80	Negative	10.34	Yes	Yes	Yes
B2	3.71	4.37	12.92	Yes	Yes	Yes
B3	4.03	Negative	0.0	Yes	No	No
B4	Negative	Negative	NT	No	Yes	No
B5	3.68	Negative	0.0	Yes	Yes	No
B6	4.06	4.23	11.4	Yes	No	No
B7	4.43	Negative	0.0	Yes	No	No
B8	Negative	Negative	NT	No	No	No
B9	Negative	Negative	NT	No	Yes	No
B10	3.72	Negative	0.0	Yes	Yes	Yes
B11	Negative	Negative	NT	No	No	No
B12	Negative	Negative	NT	No	No	No
B13	3.71	Negative	13.01	Yes	No	No
B14	Negative	Negative	NT	No	No	No
B15	5.00	4.6	11.72	Yes	No	No

NT, Not tested.

*Inhibition of sera reactivity to IFN- β by AGE-BSA (for details, see [Fig 2](#), *G* and *H*).