WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

B2140

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EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 21 November 2003

PROPOSED REPLACEMENT OF THE 1ST INTERNATIONAL STANDARD OF HUMAN TUMOUR NECROSIS FACTOR ALPHA (HTNF-α), 87/650, WITH CANDIDATE INTERNATIONAL STANDARD OF HTNF-α, 88/786, AND PROPOSED ESTABLISHMENT OF 88/786 AS THE 2ND INTERNATIONAL STANDARD OF HTNF-α. (DRAFT).

Tony Meager and Rose Gaines Das
(National Institute for Biological Standards and Control, South Mimms, Herts., EN6 3QG).

Summary

The 1st International Standard (IS) for hTNF-α, 87/650, was established in 1991. Since then, there has been a high demand for 87/650 and by July 2003 stock numbers of ampoules had fallen to 250. It is vital to conserve this remaining stock of 87/650 for any future standardisation and traceability purposes, e.g., for use in collaborative studies to evaluate future candidate IS of hTNF-α. Therefore, replacement of 87/650 is now urgent. Since there are however large stocks of three candidate IS of hTNF-α, 88/782, 88/784, and 88/786, which were prepared about 18 months after 87/650 and were included in the 1st WHO international collaborative study to evaluate such

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The 1st International Standard (IS) for hTNF-α, 87/650, was established in 1991. Since then, there has been a high demand for 87/650 and by July 2003 stock numbers of ampoules had fallen to 250. It is vital to conserve this remaining stock of 87/650 for any future standardisation and traceability purposes, e.g., for use in collaborative studies to evaluate future candidate IS of hTNF-α. Therefore, replacement of 87/650 is now urgent. Since there are however large stocks of three candidate IS of hTNF-α, 88/782, 88/784, and 88/786, which were prepared about 18 months after 87/650 and were included in the 1st WHO international collaborative study to evaluate such

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reference preparations {Meager A & Gaines Das R. Journal of Immunological Methods 1994, 170: 1-13.}, it is proposed that, rather than prepare an entirely new candidate IS, one of the three available candidate IS should be chosen as the replacement of 87/650. Candidate IS 88/786 © contains 1.0 μg of full length, 157 amino acid, hTNF-α and, although sourced differently, is the most similar of the three candidate IS to the present 1st IS 87/650. The geometric mean specific activity of 88/786 derived from results of the reference bioassay used in the 1st WHO international collaborative study is 46,500 International Units (IU)/ μg hTNF-α. Accelerated thermal degradation studies performed on 87/650, 88/782, 88/784, and 88/786 after approximately 4 years storage showed they exhibited high stability with predicted annual loss of activity at both -20 C and -70 C of less than 0.01%. More recent potency estimations in 2002 and 2003 carried out at NIBSC have given results in accord with the estimations for the three candidate IS in the collaborative study and re-confirmed the high thermal stability of 87/650, 88/784 and 88/786. It is therefore shown that 88/784 and 88/786 remain as suitable candidate IS; one of these should replace the 1st IS. Since 88/786 contains hTNF-α with characteristics most closely similar to that in 87/650 – the hTNF-α in 88/784 has a 2 amino acid deletion at its N-terminal – it is recommended that 88/786 is the most appropriate replacement and should be established as the 2nd IS of hTNF-α.

Introduction.

From the raw assay data submitted by 20 laboratories in nine countries, the potencies of several lyophilised TNF-α and one TNF-β reference preparations were calculated. The preparation 87/650 with an assigned potency of 40,000 IU per ampoule was recommended to become the 1st IS of hTNF-α. 87/650 was established as 1st IS by the WHO ECBS at its xth meeting in 1991. Due to high sustained demand for the 1st IS, stocks are now at a low level and a replacement IS is urgently required. However, stocks of three other candidate IS remain high and it is probable that one of these could serve as the replacement for 87/650.

Recent in-house studies.

Geometric mean specific activities (Table 1) based on potency estimates in relation to the potency assigned to 87/650, 40,000 International Units (IU)/μg hTNF-α protein, for the three candidate IS have been made based on data from (a) a reference bioassay and (b) in-house bioassays of participating laboratories. {Meager A & Gaines Das R. Journal of Immunological Methods 1994, 170: 1-13.} Accelerated thermal degradation studies performed on 87/650, 88/782, 88/784, and 88/786 showed they exhibited high stability with predicted annual loss of activity at both -20 C and -70 C of less than 0.01%. However, those studies were done from 1988-1991 and therefore more recent studies to evaluate potencies of 88/782, 88/784, and 88/786 have been conducted. In 2002 the potencies of these candidate IS were determined by in-house bioassays utilizing KD4 clone21 human rhabdomyosarcoma cells, which are very susceptible to the cytotoxic activity of hTNF-α {Meager A. Journal of Immunological Methods 1999, 227: 197-198}. The raw data from these bioassays were statistically analysed and the geometric mean specific activities calculated and expressed as IU/μg of 87/650 (Table 1). Overall, these geometric mean specific activities are in broad agreement with those calculated for 88/782, 88/784, and 88/786 based upon data obtained from the reference and in-house bioassays in the 1st WHO international collaborative study (Table 1). However, the scale of the recent in-house study was

much smaller, involving one operator of a single bioassay (4 assays) compared with the international collaborative study, involving 20 participants and multiple bioassays (66 reference assays and 64 in-house bioassays). Therefore, differences in specific activities seen in individual hTNF-α preparations from the collaborative study to the recent in-house study are most likely reflected by the difference in study' scale; the mean specific activities derived from the larger international collaborative study are likely to be more precise.

Table 1. Geometric mean specific activities of candidate IS of hTNF-α and other reference preparations of hTNF-α, expressed in terms of 87/650 (40,000 IU/μg) as IU/μg x 10^{-3}

Preparation	Reference Bioassay*	In-house Bioassay*	KD4cl21 Bioassay*
88/782	83.6	67.4	69.9
88/784	67.0	65.8	**56.2**
88/786	46.5	52.6	**43.8**

*For Reference Bioassay and In-house Bioassay, the geometric mean potency estimates are the means of estimates from 18 laboratories, whereas for KD4cl21 Bioassay, they are from one NIBSC laboratory (assays performed on four independent occasions).

Subsequently, the ampouled contents of 88/786 ampoules stored at specified temperatures from 4.4.89 until 24.7.02 (13 years and 3 months) as part of the accelerated thermal degradation study were tested. In this case, potency testing was also done in KD4cl21 bioassays. The mean specific activities of 88/786 stored at -70, -20, +4, +20, +37, +45, and +56 C, expressed in terms of 87/650(40,000 IU/μg) as IU/μg x 10^{-3}, are shown in Table 2.

Table 2. Accelerated thermal degradation study for ampoules of 88/786: Geometric mean specific activities of 88/786 stored at specified temperatures expressed in terms of 87/650 (40,000 IU/μg) as IU/μg x 10^{-3}.

Temperature	***Activity***	
	Assay 1	Assay 2
-70	41.0	40.7(40.3)
-20	47.9	44.0(43.4)
+4	54.8	43.4(45.1)
+20	44.2	41.7(39.3)
+37	29.7	22.3(18.1)
+45	20.1	12.8(11.7)
+56	0.9	nd

Bracketed figures calculated using Relpot method.

These results confirm the specific activity of 88/786 hTNF-α as approximately those obtained in the collaborative study and recent in-house study (Table 1) and suggest no significant losses of activity have occurred at +20 C and below. However, significant losses of activity are

apparent at +37 (48.2%), +45 (67.0%) and +56 (98.0%) compared to ampoules stored at -20 C, the normal storage temperature.

Further, more comprehensive, studies have been recently undertaken on thermal degradation samples of 88/786, 88/784 and 87/650 to confirm the previous results and to compare the degradation rates among these three preparations. The ampouled contents of 88/786 ampoules stored at specified temperatures from 4.4.89 until 25.7.03 (14 years and 3 months) were tested for potency in relation to that of 87/650 in either human KD4cl21 (2 independent assays) or murine WEHI 164 clone 13/2F2 bioassays. The calculated results (Relpot method) are tabulated below:-

Temperature	Assay 1 (KD4) Wt. mean (95% confid)	% -150 C	Assay 2 (KD4) Wt. mean (95% confid)	% -150 C
Frozen baseline	42,500 (38,500 – 47,100)	95.5	42,200 (40,000 – 44,500)	93.8
-150 C	44,500 (41,400 – 47,900)	[100.0]	45,000 (42,700 – 47,500)	[100]
- 70 C	47,000 (43,700 – 50,500)	105.6	42,600 (36,400 – 50,000)	94.7
- 20 C	49,200 (43,700 – 55,300)	110.6	43,300 (41,100 – 45,500)	96.2
+ 4 C	46,000 (40,600 – 52,000)	103.4	43,800 (34,200 – 56,000)	97.3
+ 20 C	42,900 (40,700 – 45,200)	96.4	37,700 (35,600 – 39,900)	83.8
+ 37 C	31,300 (29,600 – 33,000)	70.3	26,300 (24,800 – 27,900)	57.8
+ 45 C	11,960 (10,760 – 13,320)	26.9	13,500 (12,700 – 13,600)	30.0
+ 56 C	530 (503 – 558)	1.2	233	0.52

KD4 bioassays gave consistent results (see Table 1), but those from the WEHI bioassays were quite variable (below), although in accord and supportive of the KD4 data. In the case of KD4 bioassays, there is no evidence for significant loss of activity at + 4 C and below. However, significant losses of activity are apparent at +37 (29.7 – 42.2%), +45 (70.0 – 73.1%) and +56 (approx. 99.0%) {also possibly an average 10% loss at +20 C} compared to ampoules stored at -150 C.

Temperature	Assay 1 (WEHI) Average (n=2)	% - 150 C
Frozen baseline	45,800	91.9
-150 C	49,850	[100]
- 70 C	56,000	112.3
- 20 C	46,650	93.6
+ 4 C	47,150	94.6
+ 20 C	59,250	118.9
+ 37 C	33,800	67.8
+ 45 C	11,650	23.4
+ 56 C	426	0.9

Based on the KD4cl21 results which indicate average percent activity remaining after approximately 5,200 days is 90.1, 64.1, 28.5 and 1.0 at +20, +37, +45, and +56 C, respectively, the reaction rate constant (k) at each temperature was calculated from the equation $Y = kx + \log 100$, where Y is the logarithm of the remaining activity, and x is the stored period (days). To calculate the predicted rate of loss of activity at -20 C (the normal storage temperature for ampoules of 88/786), Arrhenius analysis was done by plotting log k against 1/T (T = absolute

temperature in degrees Celsius) and extrapolation of the line to the X-axis at 1/253 (-20). From such analysis, it is predicted that loss of 0.01% activity at -20 C requires approximately 12 years. Thus, only 0.1% activity would be lost after 120 years at -20 C. This prediction strongly suggests that 88/786 would be suitable from a stability point of view to serve as an international standard for TNF-α.

Similar results were obtained for degradation samples of another candidate IS, 88/784, stored for 14 years and 4 months (see below).

Temperature	Assay 1 (KD4) Wt. mean (95% confid)	% -150 C	Assay 2 (KD4) Wt. mean (95% confid)	% -150 C
Frozen baseline	61,400 (55,800 – 67,500)	122.8	56,000 (52,400 – 59,900)	95.1
-150 C	50,000 (45,700 – 54,700)	[100.0]	58,900 (49,100 – 70,700)	[100]
- 70 C	50,100 (44,700 – 56,100)	100.2	59,400 (46,700 – 75,600)	100.8
- 20 C	67,000 (58,000 – 77,300)	134.0	55,700 (49,500 – 62,700)	94.6
+ 4 C	59,100 (53,500 – 65,500)	118.2	56,100 (52,400 – 60,100)	95.2
+ 20 C	49,400 (43,000 – 56,700)	98.8	47,600 (44,300 – 51,100)	80.8
+ 37 C	33,400 (30,400 – 36,800)	66.8	42,200 (39,300 – 45,200)	71.6
+ 45 C	15,700 (13,800 – 17,800)	31.4	23,600 (21,900 – 25,500)	40.1
+ 56 C	121 (99.3 – 148)	0.24	10	0.02

These results strongly suggest no loss of activity at +4 C and below, but probable/significant losses at +20 (1.2 – 19.2%), +37 (28.4 – 33.2%), +45 (59.9 – 68.6%), and +56 C (>99.75%). These losses are similar to those found for 88/786 at the specified temperatures. Average percent activity remaining after approximately 5230 days storage is therefore 89.8, 69.2, 35.8, 0.13 at +20, +37, +45, and +56 C, respectively.
(for comparison, for 88/786 degradation samples average percent activity remaining after approximately 5,200 days is 90.1, 64.1, 28.5 and 1.0 at +20, +37, +45, and +56 C, respectively).
Arrhenius plot analysis of the data for 88/784 showed that, for all intents and purposes, the annual rate of loss of activity at -20 C was the same as that calculated for 88/786, i.e. 0.01% activity loss per 12 years (see graph below). Thus, 88/784 may be stored at -20C for 120 years without appreciable loss of activity.

Finally, thermal degradation samples of 87/650, the current IS of TNF-α, were tested after storage for 15 years and 9 months. Potencies were related to that of 87/786, set at 46,500 IU (its assigned potency from the reference bioassay data in the original collaborative study; Table 1) for this exercise. The results calculated from KD4cl21 bioassays, shown below *(note that the relative potency values from frozen baseline to +20 C are close to 40,000 IU the assigned potency of 87/650 strongly supporting the proposed assigned potency of 88/786, i.e. 46,500 IU),* are similar to those obtained for both 88/784 and 88/786. Significant losses of activity are only manifested above +20 C based on the results from this one complete assay. (NB. A -150C sample was not available and therefore activities are compared to that of the -70 C sample).

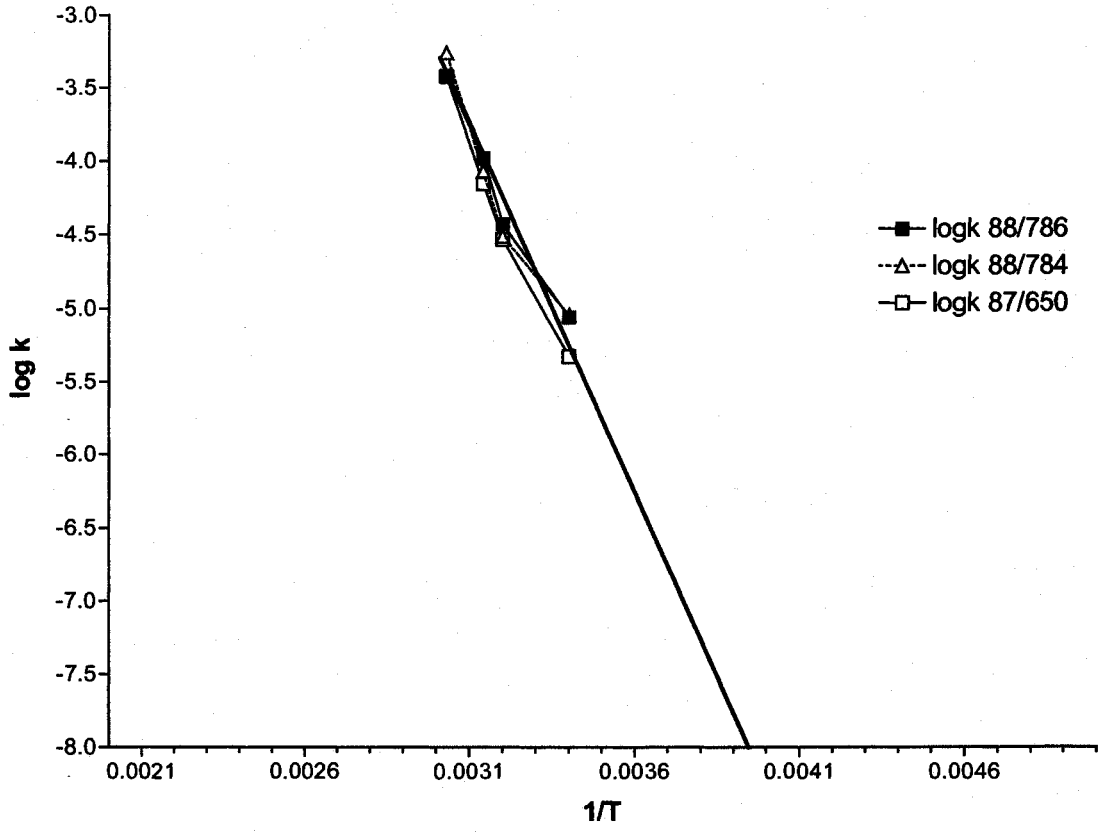
Temperature	Assay 1 (KD4) Wt. mean (95% confid)	% -70 C
Frozen baseline	41,200 (38,800 – 43,800)	101.0 (95.1 – 107.3)
-150 C	not available	
- 70 C	40,800 (38,500 – 43,300)	[100.0] (94.3 – 106.1)

- 20 C	39,500 (37,000 – 42,300)	96.9 (90.6 – 103.6)
+ 4 C	38,000 (36,200 – 39,900)	93.2 (88.7 – 97.8)
+ 20 C	38,400 (36,800 – 40,000)	94.0 (90.1 – 98.0)
+ 37 C	27,700 (22,900 – 33,600)	68.0 (56.0 – 82.0)
+ 45 C	16,100 (15,100 – 17,200)	39.5 (37.1 – 42.2)
+ 56 C	271	0.67
Temperature	Assay 2 (KD4) Wt. mean (95% confid)	% -70 C
Frozen baseline	41,800 (35,800 – 48,800)	102.3 (87.6 – 119.4)
-150 C	not available	
- 70 C	40,900 (39,400 – 42,600)	[100.0] (96.3 – 104.0)
- 20 C*	39,600 (38,100 – 41,100)	96.7 (93.1 – 100.5)
+ 4 C	39,100 (35,100 – 43,500)	95.6 (85.9 – 106.4)
+ 20 C	36,100 (32,500 – 40,000)	88.2 (79.5 – 97.7)
+ 37 C	26,600 (23,100 – 30,700)	65.1 (56.4 – 75.1)
+ 45 C	15,400 (13,500 – 17,500)	37.6 (33.0 – 42.9)
+ 56 C	130 (115 – 147)	0.3 (0.3 -0.4)

*A second ampoule of 87/650 stored at -20 C, but not included as part of the accelerated degradation study, was also titrated in assay 2. The calculated potency was 44,000 (42,400 – 45,800); 107.7% (103.6 – 11.9%) of -70 C potency. It would therefore be unlikely that activity is lost at -20C.

Arrhenius plot analysis of the data for 87/650 showed that, for all intents and purposes, the annual rate of loss of activity at -20 C was the same as that calculated for 88/786, i.e. 0.01% activity loss per 12 years (see graph below).

Arrhenius plot analysis of temperature dependent loss of lyophilised TNF-α stds (87/650, 88/786, & 88/784) activity



In conclusion, all of the hTNF-α preparations tested remain highly active and exhibit long-term thermal stability. However, since stocks of ampoules of 87/650 are very low, it is proposed that it be replaced as soon as possible with one of the candidate international standards. Of the latter, 88/786 appears the most suitable replacement since the hTNF-α it contains is structurally identical to that contained in 87/650 and its specific activity is close to that of 87/650. A potency assignment of 46,500 IU is proposed for 88/786.

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