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Development of a Lyophilization Formulation that Preserves the Biological Activity of the Platelet-inducing Cytokine Interleukin-11 at Low Concentrations

CHRISTOPHER PAGE, PETER DAWSON, DAVID WOOLLACOTT, ROBIN THORPE AND ANTHONY MIRE-SLUIS

Division of Immunobiology, NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire

Abstract

Recombinant human interleukin-11 (rhIL-11) is a licensed biological therapeutic product in at least one country and is used to combat thrombocytopenia during chemotherapeutic regimens, as well as undergoing clinical trials for a range of other disorders. Following attempts to lyophilize IL-11 at low concentrations, it was clear that a significant loss of recoverable biological activity occurred.

Investigation of a variety of factors, including the type of container in which the rhIL-11 was lyophilized, revealed that surface adsorption to glass was a major factor resulting in loss of activity of rhIL-11 in solution ($> 40\%$ reduction after 3 h at room temperature), in addition to losses of activity post-lyophilization. To overcome this problem, different formulations containing combinations of human serum albumin (HSA), trehalose and Tween-20 have been investigated.

Two formulations were successful in entirely preserving the biological activity of rhIL-11 through lyophilization and subsequent reconstitution (potency estimates of formulated relative to original material being $\geq 0{\cdot}97$). Accelerated degradation studies, performed at intervals over a six-month period, demonstrated the stability of freeze-dried rhIL-11 using these formulations (predicted annual reduction in potency after storage at $-20^{\circ}C \leq 1{\cdot}4\%$).

In conclusion, we have developed a working combination of excipients (0·5% HSA, 0·1% trehalose and 0·02% Tween-20 in potassium phosphate buffer (pH 7·4)) to formulate a stable rhIL-11 freeze-dried product in glass containers, with no loss in potency. These findings should facilitate development of low dose rhIL-11 products and be an indicator of caution to those using this and other material with similar physical properties, without taking appropriate precautions to avoid losses through adsorption.

Initially characterized as a haematopoietic cytokine with thrombopoietic activity, subsequent studies have revealed the multi-functional nature of interleukin-11 (IL-11), affecting a diverse set of cell types, mediating pleiotropic effects on multiple tissues both in-vivo and in-vitro (Du & Williams 1994, 1997; Quesniaux 1994; Yu-Chang 1995; Leng & Elias 1997). To date, the physiological role of IL-11 remains to be understood, but its expression pattern and distribution suggest that it may be involved in regulating the bone marrow microenvironment, neural and testicular function, certain inflammatory responses and bone remodelling in health and/or disease.

Recombinant human IL-11 (rhIL-11) is being developed as a biopharmaceutical and is under evaluation in human clinical trials. The haematopoietic effects of rhIL-11 have prompted studies to evaluate its therapeutic effect in the treatment of patients suffering thrombocytopenia. Findings suggest that IL-11 will be a useful thrombopoietin (Kaye 1996; Du & Williams 1997) and an rhIL-11 product (oprelvekin; Neumega – Genetics Institute) has now been approved by the US Food and Drug Administration for chemotherapy support. The anti-inflammatory effects of rhIL-11 indicate a possible

Correspondence: C. Page, Division of Immunobiology, NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire.

use in the treatment of inflammatory diseases (Maier et al 1993; Keith et al 1994; Qui et al 1996; Hermann et al 1998).

Purified recombinant proteins are sensitive to degradation and several forms of instabilities can compromise the integrity of the rhIL-11 molecule in solution. Freeze-drying has been established as a standard method for the stabilization of such materials, for use as biochemical reagents or therapeutic preparations (Hanson & Rouan 1992). This relies on the development of ''tailor-made'' formulations, taking account of specific degradation pathways as a function of pH, temperature and time to prevent chemical and physical changes, aggregation, adsorption and loss of biological activity, and to provide long-term stability on storage. To this aim, various stabilizing excipients have been described including sugars, amino acids, surfactants and fatty acids (Skrabanja et al 1994; Timasheff & Arakawa 1997). Excipients frequently used for the stabilization of cytokines include the non-reducing sugar, trehalose, human serum albumin and a range of salts and buffers, depending on the nature of the cytokine (Mire-Sluis et al 1998).

This study was undertaken to design a formulation to develop a stable, potent, freeze-dried rhIL-11 product. The findings from physicochemical pre-formulation studies were considered in the design of appropriate formulations. A cell culture based potency assay was used to investigate maintenance of rhIL-11 biological activity during formulation and the freeze-drying process. Ultimately, the conditions required to stabilize the biological activity of rhIL-11, in a fully retrievable form upon reconstitution of the freeze-dried protein were defined. This will assist in the development of stable international reference reagents and standardized rhIL-11 products, for use as a biochemical reagent or therapeutic agent, to further characterize the biological activity of IL-11 in-vitro, in-vivo and in clinical trials.

Materials and Methods

Reagents

D-(+)-Trehalose dihydrate was purchased from Fluka Biochemika (Switzerland). Tween-20 (polyoxyethylenesorbitan monolaurate) was purchased from the Sigma Chemical Company Ltd (Poole, UK). Di-potassium hydrogen phosphate and di-hydrogen potassium phosphate were purchased from BDH Chemicals Ltd (Poole, UK). Human serum albumin (HSA) (Zenalb 20. Human albumin 20% solution) was obtained from BPL Bioproducts Laboratory (Hertfordshire, UK). Recombinant human interleukin-11 (rhIL-11) was kindly donated by Genetics Institute (Massachusetts) and stored at −70°C until use. AquaSilTM Siliconizing Fluid was purchased from Pierce and Warriner (UK) Ltd (Chester, UK). Anti-IL-11 monoclonal antibodies, 19.6.1 and 15.6.13, were kind gifts from J. Wright, Genetics Institute.

Preparation of formulations containing rhIL-11 for freeze-drying

Formulations (92/522, 92/788 and 98/528A–H) containing rhIL-11 at 1 μg mL^{-1}, were prepared in potassium phosphate buffer (pH 7·4), containing 61 mM di-potassium hydrogen phosphate and 10 mM di-hydrogen potassium phosphate, made up in sterile distilled H_2O or normal saline (150 mM), containing HSA and/or trehalose and/or Tween-20 (Table 1). All excipients were 0·2-μm filter sterilized, before adding the rhIL-11 component.

Table 1. Recombinant human interleukin-11 formulation excipients.

Code	Formulation excipients									
	RhIL-11 (1 μg mL^{-1})	Phosphate buffer	Water	Saline (0·9%)	HSA		Trehalose			Tween-20 (0·02%)
					0·2%	0·5%	0·1%	0·2%	1%	
92/522	+	+		+	+		+			
92/788	+	+		+		+		+		
98/528A	+	+	+			+	+			
98/528B	+	+		+		+	+			
98/528C	+	+	+						+	
98/528D	+	+		+					+	
98/528E	+	+	+			+	+			+
98/528F	+	+		+		+	+			+
98/528G	+	+	+						+	+
98/528H	+	+		+					+	+

Freeze-dried rhIL-11 samples
Samples (1 mL) of formulations containing rhIL-11 at $1\,\mu g\,mL^{-1}$ were dispensed into sterile glass ampoules (acid washed and baked at 250°C for 1 h before filling). The ampoules were fitted with a capillary plug (Phillips et al 1991) and placed on the shelf of a freeze-drier. The temperature was lowered to −40°C, at the rate of approximately $-1°C\,min^{-1}$. The material was then freeze-dried for 116 h as follows: the shelf temperature of the freeze-drier was maintained at −40°C for 90 h, raised over a 20-h period under controlled heating to +20°C, then maintained at +20°C for 6 h. Throughout the cycle the condenser temperature was between −57°C and −59°C and the vacuum between 4×10^{-2}–$2{\cdot}5 \times 10^{-2}$ mbar. At the end of the freeze-drying cycle the ampoules were backfilled with dry nitrogen gas (O_2 level ≤ 10 ppm, $H_2O \leq 5$ ppm) and further desiccated in a vacuum over phosphorous pentoxide for six days at room temperature (20°C). They were again backfilled with dry nitrogen gas and sealed by fusion of the glass. The ampoules were stored at −20°C.

Frozen baseline rhIL-11 samples
Samples (1 mL) of formulations containing rhIL-11 at $1\,\mu g\,mL^{-1}$, were dispensed into sterile glass ampoules (acid washed and baked at 250°C for 1 h before filling), heat-sealed and stored at −20°C until use.

Biological assay
The biological activity of original rhIL-11 and formulated rhIL-11 (freeze-dried and frozen baseline samples) was assessed in-vitro, measuring proliferation of the IL-11 responsive murine plasmacytoma cell line T10, by uptake of tritiated thymidine into DNA (Wadhwa et al 1995). All standard curves were run in duplicate in different positions across three 96-well microtitre plates (six replicates per data point), to allow for inter-plate variation.

Siliconizing glass ampoules
Glass ampoules were siliconized by immersion in AquaSilTM Siliconizing Fluid, according to the manufacturer's instructions.

Adsorption study
Fixed volumes of a single preparation of IL-11 at $1\,\mu g\,mL^{-1}$ were incubated at room temperature for up to 4 h in glass, siliconized glass and polypropylene ampoules. At intervals (15, 30, 60, 120, 180 and 240 min) the concentration of IL-11 was measured by ELISA. Time dependent adsorption of IL-11 onto the ampoule surface was indicated by a reduction in the concentration of IL-11 below $1\,\mu g\,mL^{-1}$.

ELISA
Anti-IL-11 monoclonal antibodies 19.6.1 and biotinylated 15.6.13 were respectively used for capture and detection, using the method described by Meager (1995).

Stability/accelerated degradation studies
Ampoules containing freeze-dried rhIL-11 preparations were stored at −70, −20, +4, +37 and +56°C. The biological activity was measured in-vitro, using the T10 cell line bioassay, after one, three and six month storage at these temperatures, and compared with frozen baseline rhIL-11 measurements. Dose–response curves for samples stored at each temperature were run in duplicate wells across different positions on three 96-well plates (i.e. six replicates per data point), thus allowing for inter- and intra-plate variation.

Statistical analysis
Upper and lower response limits were determined from plate mean responses and used to transform raw responses to logit responses for analysis, using an in-house program (Gaines Das & Tydeman 1982). Formulation potency estimates for frozen baseline and freeze-dried material were calculated relative to original activity. Accelerated degradation potency estimates for freeze-dried material were calculated relative to frozen baseline activity. Based on within assay replication the 95% confidence limits for these estimates are approximately ±12% of the potencies. Assuming that the relationship between remaining activity and temperature can be described by Arrehenius equation, the predicted annual percentage reduction in potency was calculated using an in-house program (Kirkwood & Tydeman 1984).

Results

RhIL-11 reconstitution time course
The biological activity of two independently freeze-dried rhIL-11 preparations, 92/522 and 92/788 (formulations detailed in Table 1), was

compared with original rhIL-11 stock, for the capacity to stimulate murine T10 cell proliferation (Figure 1). The activity of both preparations was examined immediately following reconstitution (time 0), after standing at room temperature for 5 or 24 h and following incubation at 37°C for 24 h. Both freeze-dried preparations exhibited a significantly reduced activity at time 0, as indicated by the displacement of parallel dose–response curves to the right of that produced by the original unadulterated IL-11 stock. The activity of preparation 92/522, but not 92/788, was further reduced in a time and temperature dependent fashion (Figure 1).

RhIL-11 adsorption studies

The original rhIL-11 stock was stored in polypropylene and transferred into glass for freeze-drying. The following experiments were performed to elucidate whether the loss in activity of freeze-dried rhIL-11 material, as compared with original stock, was due to a difference in the rate and degree of protein adsorption to glass, as compared with polypropylene. The findings suggested a significantly greater rate of adsorption of IL-11 onto glass compared with polypropylene (Figure 2). In untreated and siliconized glass, the concentration of IL-11 decreased comparably, in a time dependent fashion. After 3 h at room temperature, residual IL-11 concentrations in glass ampoules were less than 600 pg mL^{-1} (suggesting more than 40% of the IL-11 applied had adsorbed). By comparison, the concentration of IL-11 in polypropylene did not fall below 950 pg mL^{-1} over the 4-h period (suggesting a maximum of 5% adsorption).

Comparing different formulations for their capacity to preserve the biological activity of rhIL-11 for reconstitution after freeze-drying

Eight rhIL-11 formulations (98/528A-H) containing different combinations of excipients (detailed in Table 1) were investigated. Graphical and statistical examination revealed no gross anomalies or irregularities in dose–response curves. Transformed data for each assay gave log dose–response lines with no significant deviation from linearity and parallelism.

Potency estimates for the frozen baseline relative to the original material indicate a significant loss of activity for each formulation except E and F (Table 2). Formulations without HSA and Tween-20 (C and D) lost the greatest activity when placing the solutions in glass ampoules, without any further treatment. However, the activity in formulations C and D was comparable before and after freeze-drying.

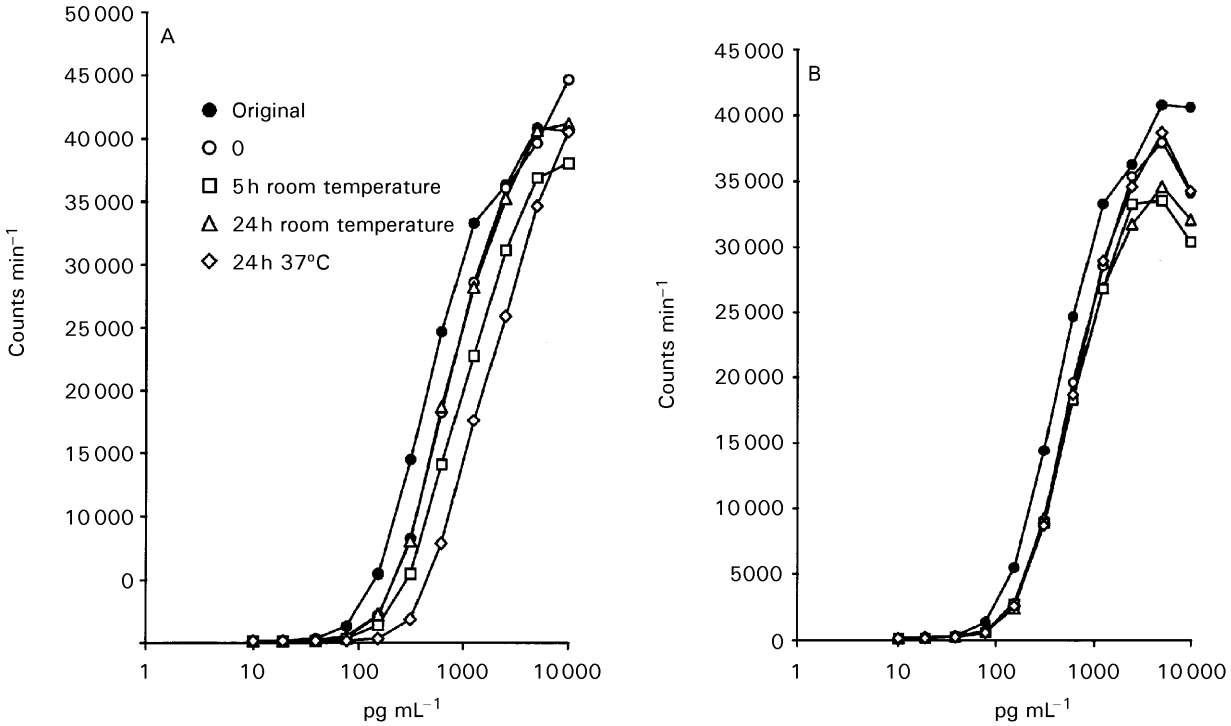

Figure 1. Comparing the biological activity of two independently formulated rhIL-11 freeze-dried preparations (A) 92/522 and (B) 92/788, with original rhIL-11 material, using the T10 cell line proliferation bioassay. The figure shows the activity immediately following reconstitution (time 0), after standing in ampoules at room temperature for 5 or 24 h and after incubation at 37°C for 24 h. Each data point represents the arithmetic mean value of six replicates.

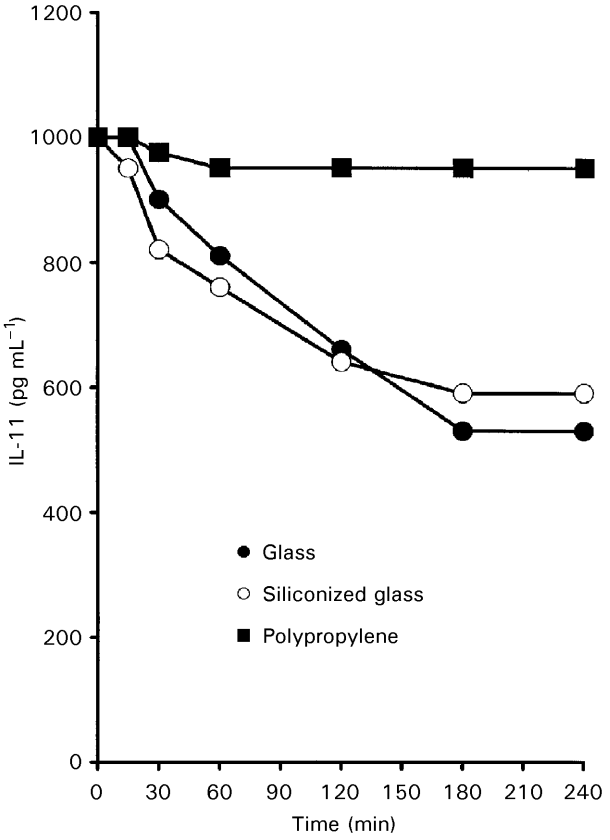

Figure 2. Comparing the residual concentration of a single 1 μg mL^{-1} rhIL-11 preparation at intervals over a 4-h period at room temperature in glass, siliconized glass and polypropylene, as determined using specific ELISA. Each data point represents the arithmetic mean value of six replicates.

Although formulations G and H (containing Tween-20 but lacking HSA) did not lose as much activity before freeze-drying as C and D, they exhibited the poorest capacity to retain the biological activity of IL-11 after freeze-drying (Table 2 and Figure 3). Preservation of retrievable rhIL-11 biological activity was significantly improved in formulations A and B (containing a combination of HSA and trehalose), to a level comparable before and after freeze-drying, indicated by small displacements of the curves in response to both frozen baseline and freeze-dried preparations (Figure 3).

Complete preservation of rhIL-11 biological activity, in a fully retrievable form following reconstitution of the freeze-dried product, was achieved using formulations E and F (containing a combination of HSA, trehalose and Tween-20).

Accelerated degradation studies to evaluate time- and temperature-dependent stability

Experiments were performed to investigate the stability of freeze-dried rhIL-11 and availability of biologically active rhIL-11 on reconstitution of formulations 98/528E and 98/528F, after storage for up to six months, at temperatures ranging from -70°C to $+56$°C. Potency estimates, relative to the frozen baseline material are shown in Table 3.

Both 98/528E and 98/528F samples stored at -70°C and -20°C showed no significant difference from frozen baseline potency, or from one another, over the six-month period. At higher temperatures ($+4$, $+37$ and $+56$°C), however, both formulations exhibited reduced potency. As expected, the samples were least stable at $+56$°C.

Predicted annual percentage reduction in potency, after storage at -20°C and $+4$°C, for 98/528E were 1·456% and 7·109%, respectively, and for 98/528F were 0·631% and 4·362%, respectively. These data suggest that 98/528F was the most effective formulation to stabilize rhIL-11 for long-term storage.

Discussion

A lyophilized preparation of IL-11 was originally developed during drug development, for doses

Table 2. Formulated recombinant human interleukin-11 (98/528A–H). Potency estimates for frozen baseline and freeze-dried preparations, relative to the activity of the original material. All preparations contain IL-11 at 1 μg mL^{-1}.

Formulation	Potency estimates	
	Frozen baseline	Freeze-dried
98/528A: IL-11, phosphate, H_2O, 0·5% HSA, 0·1% trehalose	0·73	0·67
98/528B: IL-11, phosphate, saline, 0·5% HSA, 0·1% trehalose	0·53	0·62
98/528C: IL-11, phosphate, H_2O, 1% trehalose	0·20	0·23
98/528D: IL-11, phosphate, saline, 1% trehalose	0·16	0·18
98/528E: IL-11, phosphate, H_2O, 0·5% HSA, 0·1% trehalose, 0·02% Tween-20	1·07	1·04
98/528F: IL-11, phosphate, saline, 0·5% HSA, 0·1% trehalose, 0·02% Tween-20	1·10	0·97
98/528G: IL-11, phosphate, H_2O, 1% trehalose, 0·02% Tween-20	0·80	0·33
98/528F: IL-11, phosphate, saline, 1% trehalose, 0·02% Tween-20	0·78	0·22

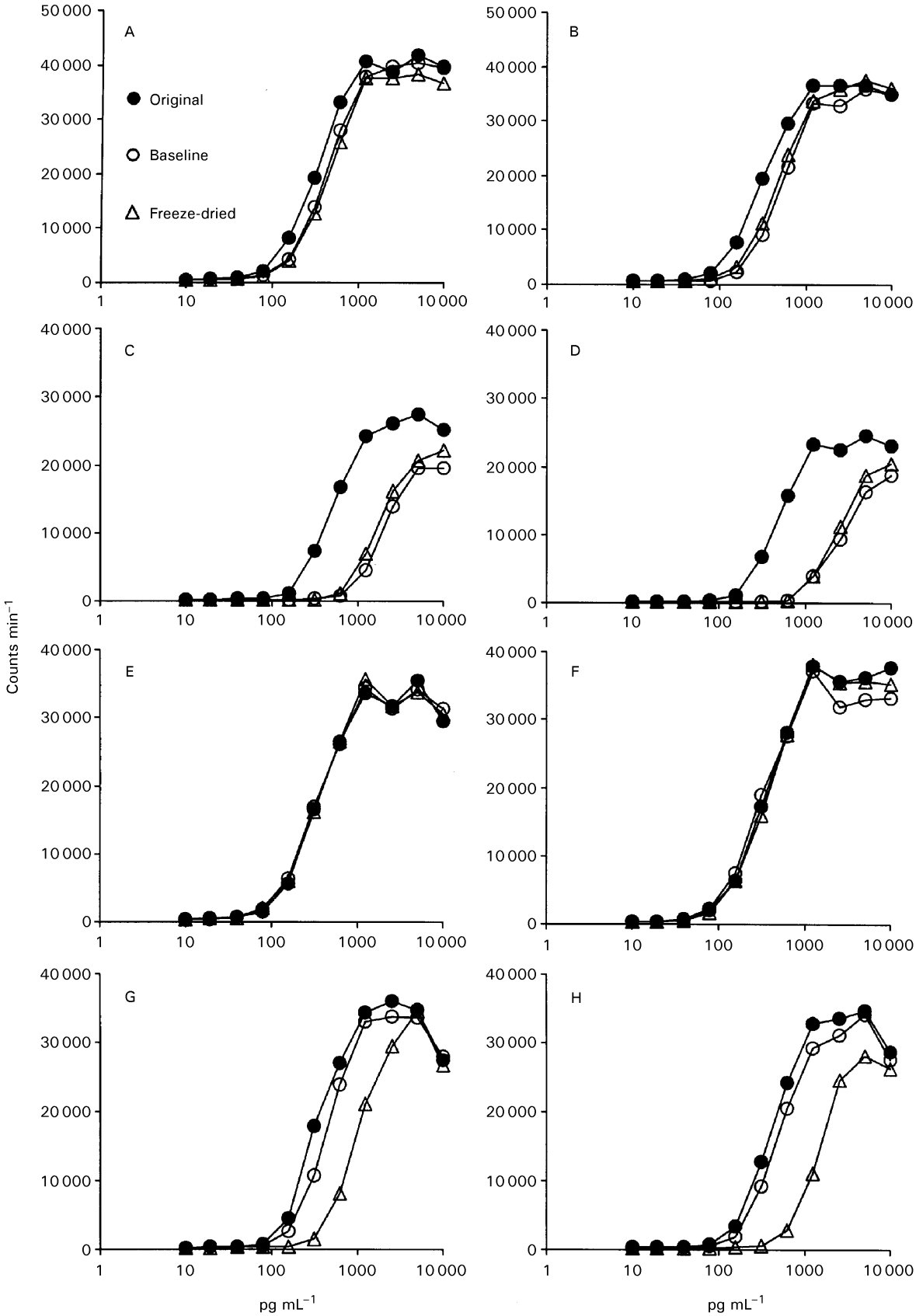

Figure 3. The biological activity of eight rhIL-11 formulations, 98/528A–H. Both frozen baseline and freeze-dried preparations for each formulation were compared with activity of original unadulterated material, using the T10 cell line proliferation assay. Each data point represents the arithmetic mean value of six replicates.

Table 3. Accelerated degradation study potency estimates for freeze-dried recombinant human interleukin-11 formulations 98/528E and F, relative to frozen baseline activity.

Formulation	Storage time (months)	Storage temperature (°C)				
		−70	−20	+4	+37	+56
98/528E: IL-11 1 $\mu g\,mL^{-1}$, phosphate buffer, H_2O, 0·5% HSA, 0·1% trehalose, 0·02% Tween-20	1	0·89	1·14	1·19	1·07	0·90
	3	0·95	0·99	0·89	0·84	0·66
	6	1·02	0·98	0·87	0·84	0·62
98/528F: IL-11 1 $\mu g\,mL^{-1}$, phosphate buffer, saline, 0·5% HSA, 0·1% trehalose, 0·02% Tween-20	1	0·85	0·80	0·98	0·88	0·70
	3	1·31	1·03	1·15	1·00	0·85
	6	1·03	0·98	1·08	0·85	0·61

containing up to milligram quantities of material. However, following clinical trials and the refinement of dosing regimens, it became clear that low dose IL-11 may be required. On investigation of freeze-drying low concentrations of IL-11 it was noted that there was significant loss of biological activity (Thorpe et al 1999).

This study shows that one area for loss of activity is the adsorption of IL-11 to the walls of glass containers, before the lyophilization process (Figure 2). Siliconization of the glass surface was unable to prevent the adsorption from occurring (Figures 1 and 2) and the subsequent loss of biological activity (Table 2). Since there is finite space on a solid surface, addition of a high concentration of other protein(s), to compete for available adsorption sites should be a useful approach to prevent the loss of low concentration recombinant proteins. Indeed, this principle explains the marked improvement in the potency of rhIL-11 in formulations containing HSA. However, addition of large relative proportions of HSA was, alone, insufficient to prevent loss of activity through adsorption. This phenomenon may be caused by the unusual amino acid composition of IL-11, which contains 23% leucine and gives the protein considerable hydrophobicity.

The inclusion of a detergent, such as the Tween-20 used in this study, has been shown to be valuable in the development of formulations for other materials. It is interesting to note that although able to reduce the loss of biological activity of IL-11 through adsorption (as seen in the frozen baselines in Table 2), in the absence of HSA, Tween-20 appeared to contribute to a dramatic loss of biological activity on freeze-drying.

Therefore it appears that a combination of HSA and Tween-20 is required to address both the adsorption of IL-11 to glass surfaces and the retention of biological activity post-lyophilization. A working combination of excipients, to formulate a stable rhIL-11 freeze-dried product, comprises a triple combination of 0·5% HSA, 0·1% trehalose and 0·02% Tween-20 in potassium phosphate buffer (pH 7·4). The findings of accelerated degradation suggest that rhIL-11 formulated using this combination will remain stable throughout adverse conditions such as elevated temperature. This is an important consideration in the transport and distribution of freeze-dried recombinant proteins.

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