INTERNAL MEMORANDUM

TO: Dr J D Cash
FROM: Dr D S Pepper

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DETECTION OF HEAT INDUCED DAMAGE TO FVIII

BACKGROUND

Heat treated FVIII:C concentrates are expected to be safer than existing products due to viral inactivation (either dry-heated at 60°C/10 hrs or wet heated under similar conditions in the presence of additives), but may not be safer in terms of ability to produce reactions as a result of neo-antigens exposed by the denaturation of protein or chemical derivatisation by additives.

Excluding for the time being the direct empirical approach of infusing product directly into patients and observing the ensuing reactions (if any) and/or assaying the patients' sera for newly arising antibody specificities, the possible techniques for prior assessment in vitro fall into three main groups, namely Physico-chemical, Immunological and Biological. Their respective advantages and disadvantages are discussed in detail below.

PHYSICO-CHEMICAL TECHNIQUES

1 Electrophoresis. Can handle large numbers of samples, uses very small volumes and monitors all protein components but small amounts (eg FVIII ≤ 1%) will not be seen. The technique can be modified (cf Ruggeri) to stain specifically for FVIII:Ag multimers but requires special expertise to develop this routinely. The use of conventional SDS is probably unrewarding since this induces general denaturation of all proteins uniformly. Paired samples before/after heating desirable.
2 Gel filtration/Ion Exchange on HPLC. Can handle multiple samples (but not as many as electrophoresis) and uses small sample volumes. Resolution of individual bands is less than electrophoresis but is more suitable for detection of aggregates and is quantitative or semi-quantitative. Already used by FFC for IgG and albumin.

3 Nephelometry/Laser Light Scattering. The former is used by FFC, the latter is available via the author. Nephelometry is a qualitative/semi-quantitative technique particularly useful for aggregate detection. Laser light scattering is simply a quantitative version of the same technique in that it gives the absolute size and distribution of molecules and aggregates. Sample volumes are large and numbers (in the latter technique) are limited. Complements gel filtration in (2) above.

4 Chemical. There exists a slight possibility that during heating, new chemical bonds are formed between sugars (eg sorbitol) and amino acids (eg glycine) and the existing proteins to give neo-glycoproteins. In practice the risk is small because a non-reducing sugar and neutral pH are used. It might be feasible to look for sugar/amino acid incorporation by the use of ³H-radioactive labels in the sorbitol and/or glycine moieties. Spectrophotometry/fluorescence may also be used to detect gross changes in molecular shape in purified proteins, but are less likely to be suitable with impure mixtures.

IMMUNOLOGICAL TECHNIQUES:

5 Parallélism with Polyclonal RIA's. Given that existing RIA's for various major constituents of FVIII concentrate are running, work has already been performed with the antigens FVIII:Ca, FVIII:Ag, Fibrinogen, 8-TG, PF4, TSP and could in future be done with fibronectin, FIX and t-PA. The sample volume is small but the number of samples is limited and the technique is inherently labour intensive. Its
major drawback, however, is that damage will only be seen in the analyze
understudy and also only if it results in a shift in slope (non-
parallelism) of the heated analyze relative to the unheated. So far
no changes in slope have been seen.
Physical problems with the fibronectin assay have not been solved and a
major development input seems unjustified given the small chance of
this being a universal test of damage

Parallelism with Monoclonal Antibodies. This technique is identical to
that described in (5) above but uses a panel of McAb's which are directed
against different specific epitopes on a given molecule. Currently
available McAb's include specificities to FVIII:Ag, CAg, Fibrinogen,
t-PA and FIX, though only the first two are likely to be of use.
Dr Prowse has already carried out parallelism studies using McAb's
to FVIII:Ag and it is proposed that Brenda Griffin do a similar study
with FVIII:CAg McAb's. So far, no changes have been detected.

BIOLOGICAL TECHNIQUES

Existing Bioassays can be put to good use. These include FVIII:C
clotting and/or micro-chromogenic assay, FVIII-Ristocetin co-factor
activity particularly when related to total protein and total antigen
contents, i.e. specific activity.

Non-specific Chromogenic Substrates for Proteases (such as S-2238)
can be used to monitor increases (activation) or decreases
(denaturation) in serine proteases which are known to occur in FVIII
concentrates. Possible candidates include thrombin, FIX, FX, t-PA,
kallikrein. Likely to be insensitive because levels are already
deliberately low in process of manufacture.

Future Bioassays. It would be prudent to collect patients sera prior
to and following exposure to heated products, thus formation of potential
inhibitory antibodies could be monitored in "Bethesda" type clotting assays or similar chromogenic assays.

CONCLUSIONS

No one assay clearly stands out as being suitable, all are compromised to a greater or lesser extent, but of the existing assays, I feel that categories 1-4 are most practicable.