ABILITY OF THE ISOELECTRIC FOCUSING TECHNIQUE TO DISTINGUISH BETWEEN STRUCTURALLY DIFFERENT IMMUNOGLOBULINS

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1. Introduction

Isoelectric focusing (IEF) in acrylamide gel slabs [1] can distinguish between different immunoglobulin molecules on the basis of differences in their isoelectric points. The resolution of the system for mouse immunoglobulins is high; thus, Kreth and Williamson [2] examined the products of over 300 clones producing anti-NIP antibody, and found only 5 pairs of indistinguishable antibodies ('overlaps') on making all possible comparisons between IEF spectra of the clones. Similarly, Pink and Askonas [3] found only 5 definite overlaps in about 2400 comparisons between clones producing antidinitrophenyl-lysine antibodies cross-reactive with the trinitrophenyl hapten. Even molecules with very similar primary structures and idiotypic specificities, such as the phosphoryl-choline binding mouse myeloma proteins TEPC-15 and HOPC-8 [4], can be distinguished by this technique (J. R. L. Pink, unpublished results).

Williamson et al. [5] have given arguments (based on parameters such as the observed average number of bands seen after focusing of a monoclonal immunoglobulin, the variability in spacing between them, and the width of one band; see Results and discussion) that the IEF technique can resolve as many as 50 000 different mouse immunoglobulins. The power of the technique, however, is in practice determined by the probability that two different immunoglobulins will appear to be identical in IEF pattern. This probability would be the reciprocal of the number of resolvable patterns, i.e. 1/50 000, if all antibody patterns occurred with equal frequency. Since this is most unlikely to be the case, the ability of IEF to distinguish different immunoglobulins must be evaluated empirically. To our knowledge, this has not been done for immunoglobulins of any species.

Here we have investigated the resolution of IEF for human immunoglobulins by comparing the banding patterns of 45 different human myeloma proteins, and determining the parameters which affect the ability of the technique to distinguish one myeloma protein from another. We estimate that the probability of being unable to distinguish between two such proteins is of the order of 1:5000 (that is, of 5000 comparisons between structurally different immunoglobulins, one comparison would appear to be between identical proteins).

2. Materials and methods

Human myeloma sera were obtained from various clinical institutes and laboratories in Switzerland. Myeloma proteins were typed for light chain class and heavy chain subclass as described by Skvaril and Barandun [6]. In some cases, myeloma proteins were purified by Pevikon block electrophoresis followed by gel filtration through Sephadex G-200.

The sample studied consisted of 32 IgG1 myeloma protein-containing sera (18 K and 15 λ), and 12 purified IgG1 myeloma proteins (8 K and 4 λ). One serum contained two (IgG1, K) myeloma proteins. Isoelectric focusing of 5–30 μl serum samples, or 100 μg protein samples, in acrylamide gel slabs containing 1% Ampholine was performed according to Awdeh et al [1]. Groups of 10–15 sera or proteins were initially run on pH 3.5–10 gels, and then on

appropriate narrower pH gradients. Isoelectric points of the various bands given by each protein were derived from measurements of gel pH made with a flat electrode [7]. Gels were stained with bromphenol blue after determination of the pH gradient.

3. Results and discussion

Fig.1 shows that myeloma proteins can be identified after isoelectric focusing of whole sera, and that sera containing biclonal myeloma proteins or raised levels of all Ig species can be distinguished from those containing monoclonal myeloma proteins. The proteins studied focused between about pH 6 and pH 9, with a majority focusing between pH 7.5 and pH 9, as also found by other workers for whole human IgG or IgG1 subclass [8]. There was no significant difference in the distribution of K and λ chain types over this range.

The number of bands given by a single myeloma protein ranged from 2 to 9, the most common values being 4 and 5; the spacing between them varied from 0.07 to 0.17 pH units. These figures are similar to those obtained for mouse immunoglobulins [5]. The spacing between bands of a single protein is rather

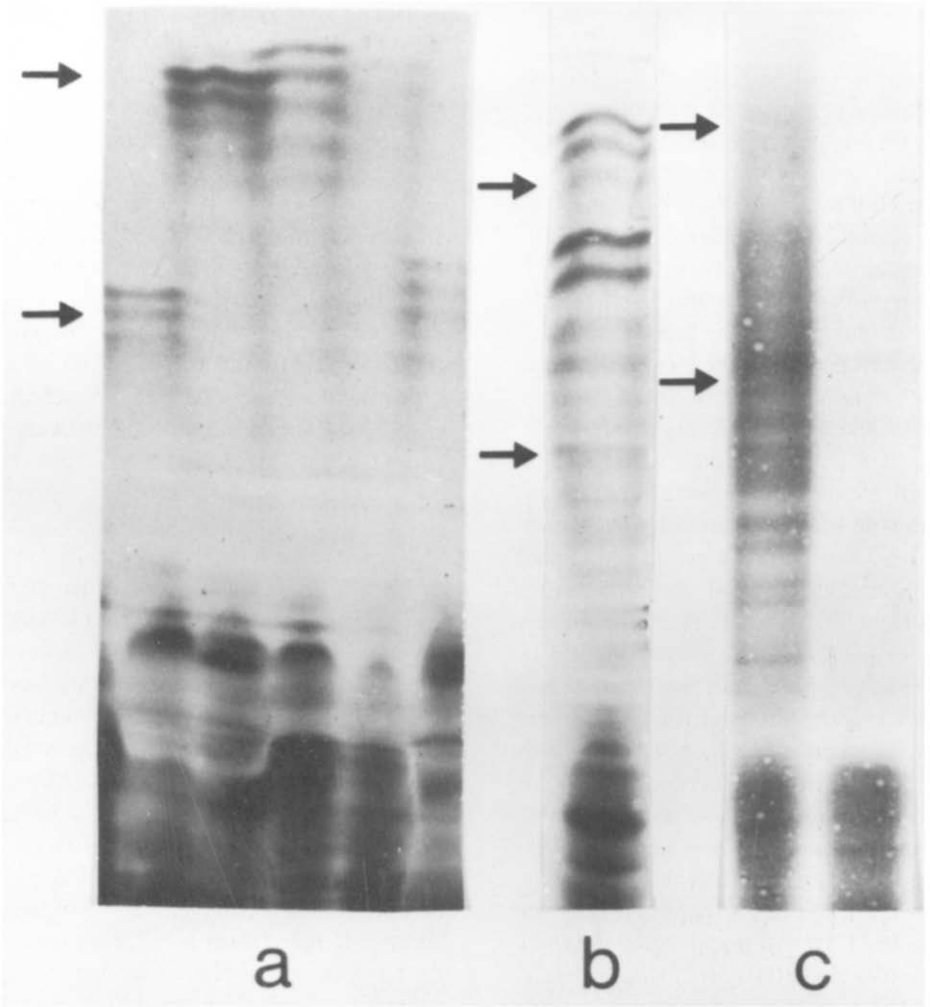

Fig.1. Isoelectric focusing patterns of 20 μl samples of (a)five myeloma protein-containing human sera; (b)serum containing two myeloma proteins; (c)hypergammaglobulinemic and normal sera. Arrows are at pH 8.5 (top) and 7.5 (bottom).

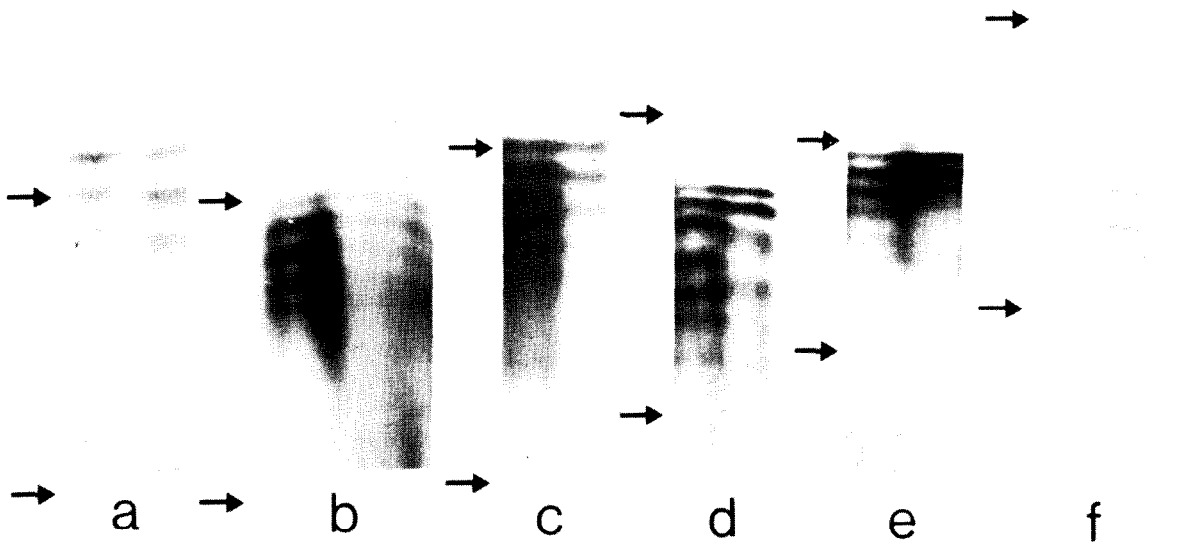

Fig.2. Resolution of isoelectric focusing for human myeloma proteins. In (a) and (b) the leading bands of the proteins being compared can be distinguished, since they focus at p*I*s which differ by about 0.01 pH units. In (c) and (d) the leading bands of the proteins being compared have indistinguishable p*I*s and in (e) and (f) the leading two bands of the proteins being compared are indistinguishable, but the p*I*s of minor bands are different in each case. Arrows are at pH 8.5 (top) and 7.5 (bottom).

regular, as also observed for mouse immunoglobulins; some examples are shown in fig.2. The width of a single band is about 0.03 pH units, so that bands can be resolved when more than about 0.01 pH units apart (fig.2).

No two proteins gave identical banding patterns. In five cases, the leading bands of two proteins coincided and in two of these cases, the second bands also overlapped; but in each case there were consistent differences in the positions of minor bands (fig.2).

The resolving power of IEF for mouse anti-hapten antibodies is known to be very high [2,3,5]. The results reported here show that the resolving power of the technique for human immunoglobulins is also high; the fact that of 45 randomly chosen IgG1 myeloma proteins (both K and λ), all gave distinctive banding patterns, suggests that the probability P of finding identical patterns when comparing such proteins is at least $45^2/2$ (or about 1000), and thus that the technique will resolve at least this number of different human IgG proteins.

Another estimate of P can be made as follows. If only the leading bands of each protein are compared, the probability of finding identical patterns is about 1:200 (since 5 cases were seen in about 1000 comparisons); if the leading two bands of each protein are considered, the probability of finding identical patterns is still quite high -- about 1 in 500. Consideration of additional (3rd, 4th, etc.) bands increases the probability of resolving structurally different proteins, but not by more than one order of magnitude [5] since the band spacings of each protein tend to be regular (fig.2). Thus we estimate that the actual probability of being unable to resolve randomly-chosen, structurally-different human immunoglobulins by IEF under the conditions described here is of the order of 1:5000 – about an order of magnitude higher than the 'best case' limit (about 1 in 30 000 for human proteins) derived* as suggested by

*The 'best case' limit or maximum number of immunoglobulins resolvable by IEF, is taken to be $r_1 \times r_2 \times 10/W^2$ where W is the p*I* difference between bands which can just be distinguished (0.01 pH units), r_1 is the pH range over which the proteins focus (3 pH units) and r_2 is the difference between maximum and minimum spacings of the leading and 2nd bands of the proteins considered (0.1 pH units). The factor 10 (a guess, see text) is introduced to allow for further resolution on consideration of the proteins' 3rd, 4th, etc. bands.

Williamson [5]. The difference is presumably due to the fact that the 'best case' limit is obtained by making some simplifying assumptions: for example, that all band spacings between the largest and smallest observed (here 0.07 and 0.17 pH units) are equally probable. Non-randomness in this distribution will increase the estimated *P*.

The resolution of IEF will of course depend somewhat on the experimental conditions employed, and more importantly, on the sort of immunoglobulin being studied. Thus there is evidence [8] that structurally different guinea-pig anti-DNP antibodies, most of which have high isoelectric points, relatively often give indistinguishable IEF patterns. However, the parameters affecting resolution in IEF of human myeloma proteins and mouse anti-NIP antibodies [5] are rather similar. In fact, since the value of *P* (1:5000) obtained for human proteins is not significantly different from the proportion of overlaps found in experiments involving comparisons of antihapten antibodies in mice (5 out of 50 000 [2] and 5 to 7 out of 2400 [3]) the work reported here suggests that the limit of resolution of the IEF method may have been reached in these experiments; and thus that the numbers of structurally different antibodies made by an inbred mouse strain to the hapten NIP [2], or the cross-reacting haptens di- and trinitrophenyl-lysine [3] may well exceed the reported minimum values of 8000 and 500 respectively.

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