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Human triclonal anti-IgG gammopathy

I. ISO-ELECTRIC FOCUSING CHARACTERISTICS OF THE IgG, IgA AND IgM ANTI-IgG AND THEIR HEAVY AND LIGHT CHAINS

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Summary. Human IgG, IgA and IgM anti-IgG autoantibodies have been isolated from the serum of an individual with Felty's syndrome. These were initially noted as soluble circulating serum complexes by analytical ultracentrifugation. Isolation was accomplished by solid phase immunoadsorption and each of the three antibody populations obtained was shown to be of restricted heterogeneity by liquid and polyacrylamide gel electrofocussing methods. Type kappa light chains were obtained from each protein. Co-isoelectric focusing experiments of all possible pairs of these light chains showed them to have identical net charge characteristics. Heavy chains obtained from each protein were also monoclonal and of differing isoelectric point.

The availability of this serum provides a human model with which to study the changes which may occur in autoantibodies during the autoimmune response.

INTRODUCTION

The simultaneous occurrence of two or three myeloma proteins, each of a different immunoglobulin class in the serum of one individual, has been described in several instances. These reports have included IgM and IgG (Wang, Wang, McCormick & Fudenberg, 1969; Penn, Kunkel & Grey, 1970), IgM and IgA (Yagi & Pressman, 1973), IgG and IgA (Fair, Krueger, Gleich & Kyle, 1974; Wolfenstein-Todel, Franklin & Rudders, 1974) and IgA, IgG and IgM (Grubb & Zettervall, 1975).

When these proteins have been characterized and compared by immunological and/or biochemical methods, the data have suggested that the heavy chain variable regions and the light chains may be nearly, if not actually, identical in structure for proteins isolated from the same serum. The experimental data derived from the studies which described these proteins have been used to explain how antibody diversity and changes of immunoglobulin class may occur during the immune response.

These proteins were isolated from the sera of patients with malignant dyscrasias and none has had a defined antigenic specificity. Further, it has not been possible to follow these proteins for prolonged periods such that the persistence of and/or the switching from, one antibody class to another may be studied during the progress of an immune response.

Serum of patients with rheumatic diseases contain, in most instances, antibody which reacts *in vivo* with the patient's own IgG. Previous studies have shown that anti-IgG antibodies or rheumatoid

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factors which comprise the three major immunoglobulin classes may be contained within and isolated from a single serum, and retain their anti-IgG activity (Heimer & Levin, 1966). Further, in a few instances, rheumatoid factors of restricted heterogeneity have also been described. Monoclonal IgM (Metzger, 1967), IgG (Capra, Winchester & Kunkel, 1971) and IgA (Abraham, Clark & Vaughan 1972) anti-IgGs have been isolated in quantities sufficient for the determination of their structural and antigenic characteristics. The occurrence of monoclonal populations of rheumatoid factors which comprise more than a single immunoglobulin class in one serum might not be unexpected.

This and following reports will describe the characteristics of IgG, IgA and IgM anti-IgG autoantibodies which were isolated from the serum of a patient (Gil) with Felty's syndrome, i.e. a triclonal rheumatoid factor gammopathy.

MATERIALS AND METHODS

Isolation of anti-IgG autoantibodies

The anti-IgG antibodies were isolated by solid phase immunoadsorption of serum followed by molecular-sieve and ion-exchange chromatography of the eluate. Lipid-free serum diluted five to ten-fold with phosphate buffered 0·15 M NaCl pH 7·4 (PBS) was acidified to pH 2·9 with 10–30 μl aliquots of glacial acetic acid and placed on a bromoacetyl cellulose human IgG-l immunoadsorbant (Robbins, Haimovich & Sela, 1967) pre-washed with 0·1 M acetic acid. The serum and BAC-IgG were dispersed for 10–15 min and slowly adjusted to near neutrality with 1 M NaOH. The serum BAC-IgG slurry was incubated at 37° for 1 h and 4° for 2 h with intermittent, gentle manual dispersion. After incubation, the mixture was centrifuged (10,000 rev/min, 20°) and washed with 10 volumes of ice-chilled 0·1 M borate buffered 0·1 M NaCl pH 7·8 (BBS) until the optical density (OD-280 nm) of the washes was less than 0·1. The pool of anti-IgG antibodies was dissociated and collected from the BAC-IgG by incubation in 0·1 M acetic acid (20–30 volumes per volume of packed BAC-IgG) at 37° for 1 h, followed by centrifugation of the adsorbant for 30 min at 13,000 rev/min (18,000 g) and 20°. The eluate was removed, the adsorbant resuspended in 0·1 M acetic acid, rewashed without incubation, and the residual anti-IgG recovered. The two eluates were pooled, passed through a single 0·45 micron or two 3·0 micron ultrafilters, and dialyzed vs 30–40 volumes of 1/2 strength, and then full-strength BBS. The eluates were concentrated by pervaporation, and placed on Sephadex G-200 (SG-200) to separate the IgM (first peak) and IgG:IgA (second peak) rheumatoid factors. The latter peak was reconcentrated, dialyzed vs 0·015 M phosphate pH 7·4, and placed on DEAE-cellulose equilibrated with 0·01 M phosphate pH 7·3. A two-step gradient of 0·01 M and 0·150 M phosphate buffers resolved the SG-200 second peak into IgG and IgA pools respectively. Column pools were assayed by immunodiffusion and immunoelectrophoresis with rabbit anti-whole human serum and rabbit antisera specific for the human light and heavy chain determinants. The anti-IgGs thus isolated produced sharp bands when assayed by analytical polyacrylamide disc gel electrophoresis. The anti-IgGs were tested for restricted heterogeneity by analytical isoelectric focusing. These experiments are described in detail below.

Prior to isoelectric focusing, the anti-IgG antibodies were trace-labelled with 0·1 molecule or less of ^{125}I or ^{131}I per molecule of protein by the iodine monochloride method described by Bale, Helmkamp, Davis, Izzo, Goodland, Contreras & Spar, 1966. For analysis of light and heavy chains, the anti-IgGs were totally reduced in 0·05 M dithiothreitol and alkylated with a 10% molar excess of iodoacetamide in 0·2 M Tris-HCl, pH 8·4, 6–8 M in urea. Heavy and light chains were obtained by fractionation of reduced and alkylated protein over a Sephadex G-100 column equilibrated with 1 M propionic acid 8 M in urea. These preparations were dialyzed against 8, 6 and 2 M urea prior to isoelectric focusing.

Isoelectric focusing

Analytical isoelectrical focusing was performed over various pH ranges by both liquid column and polyacrylamide gel techniques. The liquid isoelectric focusing procedure was performed as previously described (Trieshmann, Abraham & Santucci, 1975) except that the 40–1% sucrose gradient and electrode solutions were prepared in 1 or 2 M urea, as indicated.

Isoelectric focusing in polyacrylamide gel columns (5×130 mm) was performed as follows: 0·01 to 0·02 mg of ^{125}I trace-labelled anti-IgG antibody was

added to a solution of 15% acrylamide-bisacrylamide with one-tenth the final volume of carrier ampholyte spanning the appropriate pH range. The final gel concentration was adjusted to 7 or 4% by the addition of 4 M urea in water. Polymerization was initiated by addition of 7–10μl of TEMED (N, N, N′, N′ tetramethylethylenediamene), and 1–2 μl of freshly prepared 10% ammonium persulphate.

An anode solution of 0·5% sulphuric acid and cathode solution of 1·0% 2-aminoethanol, both 1·0 or 2·0 M in urea, were utilized. A potential of 100 V was applied for 1 h, and increased to 200 V for 5–7 h. Gels were removed from the tubes, immediately cut into 1 mm slabs and placed into 1 ml of double, glass-distilled water for 24 h at room temperature. After vortex mixing, the radioactivity and pH of each slice were determined. Approximately 10,000 to 50,000 c.p.m. of specific radioactivity were utilized for each gel. In order to minimize the background percentage of ^{131}I which counts as ^{125}I, proteins labelled with ^{125}I were adjusted to at least three times the counts of protein labelled with ^{131}I in co-isoelectric focusing experiments. In addition, the ^{125}I counts were always corrected for the non-specific ^{131}I spillover. Occasionally gels from a given run were fixed and stained in order to correlate the visual homogeneity and position of the protein band in the gel, with the measurements of radioactivity obtained in a corresponding gel. All samples were iso-focused over a similar pH range at least four times. In our hands, the isoelectric points obtained in gel varied by as much as $\pm$ 0·30 pH units between gel columns. The technique was highly reproducible as to the pattern of protein dispersion and provided consistent data by which the heterogeneity of the protein was able to be assayed. Variability by the liquid column technique for replicate samples of anti-IgG isofocused in the same column with the same ampholine solution was not more than 0·05 pH units. The use of urea in solutions in nearly all instances abolished the electrode compartment trapping which at times is marked for some rheumatoid factor of low pI. Its use produces slightly higher iso-electric points than when these same proteins are iso-focused in non-urea containing media. Nevertheless, the intact anti-IgGs reported here had acidic pI of less than pH 4·95, and no differences were noted in the span of charge restriction obtained for the electrofocusing profiles.

RESULTS

Figures 1a and b demonstrate a representative experiment of the chromatographic procedure used to separate these rheumatoid factors. In this experiment, 3 ml of Gil serum were immunoadsorbed twice on a bromoacetyl cellulose human IgG-1 adsorbant. This procedure yielded approximately 30 mg of rheumatoid factor eluate, which contained IgG, IgA and IgM with only type kappa light chains. Two-thirds of the total protein was obtained from the first and one third from the second adsorption. One-third of the concentrated eluate was fractioned on a Sephadex G-200 column and pooled as indicated in Fig. 1a. Immunodiffusion in agar gel utilizing rabbit antisera specific for human immunoglobulin heavy chain class determinants revealed that peak No. 1 contained IgM, and peak No. 2, IgG and IgA. As visualized, these peaks contained approximately 75% and 25% of the optical density units in the eluate, respectively. Figure 1b illustrates the results obtained by fractionation of the SG-200 peak No. 2 on a DEAE-cellulose column. This separation produced nearly equivalent quantities of IgG and IgA. All column pools contained potent anti-IgG activity (specificity studies presented elsewhere).

The immunoglobulin class and light chain type of the proteins were established by immunodiffusion and immunoelectrophoresis utilizing antisera specific for the human immunoglobulin heavy chain classes and light chain types. These were prepared and donated by Dr J. P. Leddy (Department of Medicine, University of Rochester School of Medicine, Rochester, New York).

Isoelectric focusing

The anti-IgGs were studied by analytical isoelectric focusing in order to determine their charge heterogeneity and net charge identity. Each preparation was isofocused at least four times in gel with duplicate or triplicate gels in each run, and three times in liquid ampholine columns. The isoelectric focusing patterns presented are very representative and were repetitively reproduced.

Each Gil-rheumatoid factor demonstrates a sharp spike, markedly restricted IEF profile and an acidic isoelectric point. The variability in pI with the gel focusing technique is shown in Fig. 2, since values of 4·62 and 4·88 were obtained for the IgG-Gil

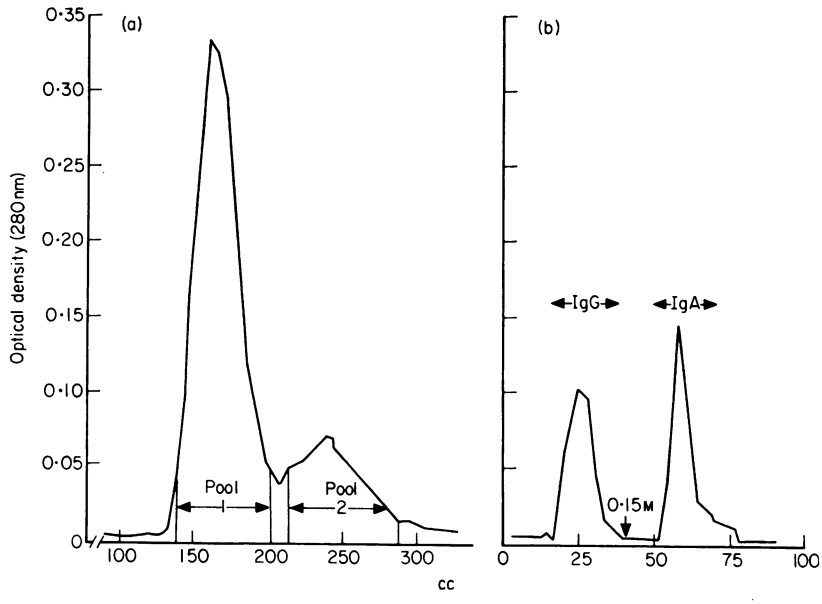

Figure 1. Sephadex G-200 and DEAE column chromatograms of Gil-eluate. One-third of eluate was placed on a Sephadex G-200 column (2·5 × 90 cm) in borate buffered 0·154 M NaCl pH 7·8; Pool No. 1 contained IgM; Pool No. 2, IgG and IgA. Pool No. 2 was dialyzed versus 0·015 M phosphate and placed on a DEAE-cellulose column (1·0 × 40 cm). IgG and IgA eluted in the positions indicated. The 0·15 M phosphate pH 7·4 buffer was started after IgG eluted. All column fractions contained potent anti-IgG activity. (a) SG-200 (b) DEAE.

rheumatoid factor when isofocused over the interval pH 3–6. By the liquid column technique, the isoelectric point of IgG-Gil was 4·89 ±0·03. These latter runs were performed in 2 M urea. An isoelectric point of 3·70 has been obtained for this anti-IgG when the same preparation was electrofocused in medium without urea.

Figure 3 illustrates representative gel electrofocusing profiles for IgM-Gil and IgA-Gil. pI at pH 4·82 and pH 4·72 were obtained for the IgM and IgA respectively and isoelectric points at pH 3·75 and 4·50 were repeatedly obtained for IgM-Gil and IgA-Gil in non-urea containing media.

In order to determine if the type kappa light chains of these proteins exhibited equivalent charge characteristics, these were radiolabelled, reduced and alkylated, and subjected in pairs to simultaneous liquid isoelectric focusing in 2 M urea sucrose-gradient columns. The IEF profiles obtained are characteristic of and similar to those previously noted for several other monoclonal myeloma proteins and anti-IgG antibodies (Abraham *et al.*, 1972; Abraham, Santucci & Jacox, 1974; Ristow, Griner, Abraham & Shoulson, 1976) whose homogeneity has been established by demonstration of a single amino-terminal amino acid sequence by automated primary amino-acid sequence analysis. Figure 4 a, b, and c illustrate prominent spikes whose peaks are identical for each protein pair. That these counts were due specifically to radiolabel on kappa chains was demonstrated as follows: Type kappa light chains were isolated from a purified IgG myeloma protein, concentrated and dialyzed to neutrality. The equivalence point was determined for the precipitation reaction between these kappa chains and an absorbed antiserum specific for kappa chain determinants. The peaks of radioactivity from the co-isoelectric focusing runs were added to the purified cold carrier kappa chains and the anti-kappa antiserum. After an appropriate incubation period, nearly 85% of the counts were precipitated at equivalence. However, only 8–10% of the counts were found in unwashed precipitates produced by human lambda chain:anti-lambda chain and human serum albumin:anti-albumin precipitation reactions. The extraneous spikes of background radioactivity noted were lost during dialysis (exclusion limit 1100 mol. wt) and were not analyzed.

The pI values noted are at pH 6·8 (IgG:IgA), pH 6·85 (IgM:IgG) and pH 6·8 (IgM:IgA) for each pair

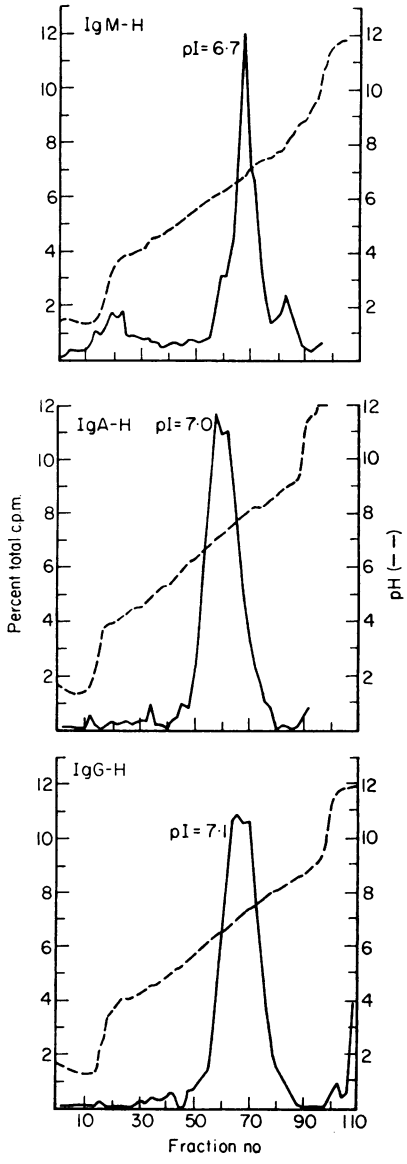

Figure 2. Isoelectric focusing profiles obtained for IgG-Gil by polyacrylamide gel and liquid column techniques over the pH range of 3–8. All media and electrode compartment solutions were 2 M in urea. All liquid columns fractionated into 101 1 ml fractions, and gels into 60–642 mm slabs for assay of protein radioactivity (solid line) and pH (broken line).

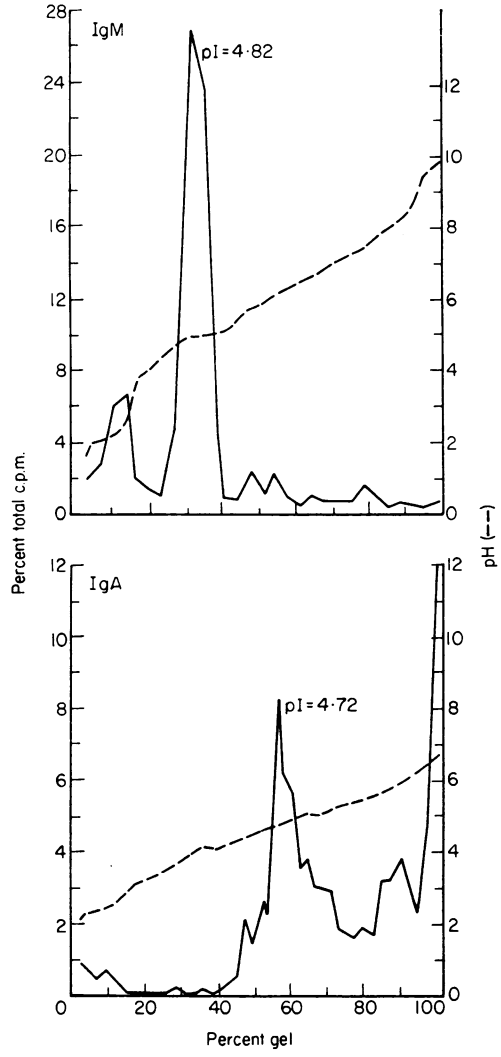

Figure 3. Isoelectric focusing profiles for the IgM-Gil and IgA-Gil in 75% polyacrylamide gel columns. Electrofocusing was performed over the pH ranges of 3–8. All solutions and the acrylamide gels were prepared in 2 M urea.

of light chains. Thus, by the criteria of identical pI, IEF profiles and marked charge restriction, the light chains of these antibodies are apparently equivalent.

Heavy chain preparations from each protein were individually isoelectric focused in liquid and polyacrylamide gel columns. No conditions were found which permitted analysis of heavy chains in 7·5 or 10% cylindrical polyacrylamide gel columns.

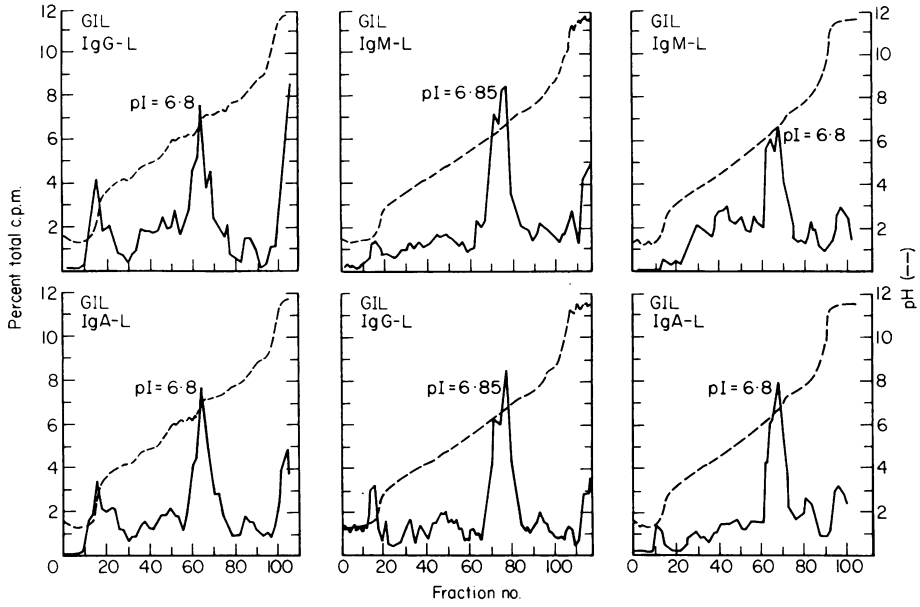

Figure 4. Electrofocusing of the kappa light chains obtained by total reduction and alkylation of IgG, IgM and IgA-Gil. The Gil-light chains were simultaneously isofocused in pairs as indicated over the pH range of 3–8, and in 2 M urea. Identical pI are noted for each pair.

These experiments were thus performed exclusively in liquid ampholine columns containing 6 M urea. Fig. 5 shows restricted profiles for the Gil IgA, IgG and IgM heavy chains, and pI at pH 7·0, 7·1 and 6·7 respectively. The restricted isoelectric focusing profiles noted are typical of monoclonal proteins.

DISCUSSION

The data present the isoelectric focusing characteristics for IgG, IgA and IgM anti-IgGs which were isolated from the serum of a patient with Felty's syndrome. Since these proteins have very limited charge heterogeneity, this is the first report which describes monoclonal proteins of known antigenic specificity and of the three major immunoglobulin classes in the serum of one individual.

When the anti-IgGs were assayed by analytical isoelectric focusing in liquid and polyacrylamide gel columns, each autoantibody produced an electrofocusing profile equivalent in charge restriction to those obtained for purified myeloma proteins analyzed by identical techniques (Abraham *et al.*, 1972; Abraham *et al.*, 1974). The acidic isoelectric points (pI) are similar to those previously noted by Trieshmann *et al.* (1975) for ten other monoclonal rheumatoid factors which were of all immunoglobulin classes.

These findings are in some respects analogous to studies in which two monoclonal immunoglobulins have been noted in the sera of patients with multiple myeloma (Wang *et al.*, 1969; Penn *et al.*, 1970; Yagi & Pressman, 1974; Fair *et al.*, 1974; Grubb & Zettervall, 1975). These previous reports demonstrated structural and/or serological identity of the light chains isolated from each individual. In order to determine if a similar situation existed for the Gil anti-IgGs, the isolated kappa chains were analyzed in co-isoelectric focusing experiments. All the possible paired combinations of these light chains produced identical and superimposable electrofocusing profiles with pI at 6·8. Thus, each antibody contains a light chain with equivalent net charge characteristics. As will be shown in another report, the kappa light chain of IgM-Gil contains an amino-terminal amino acid sequence character-

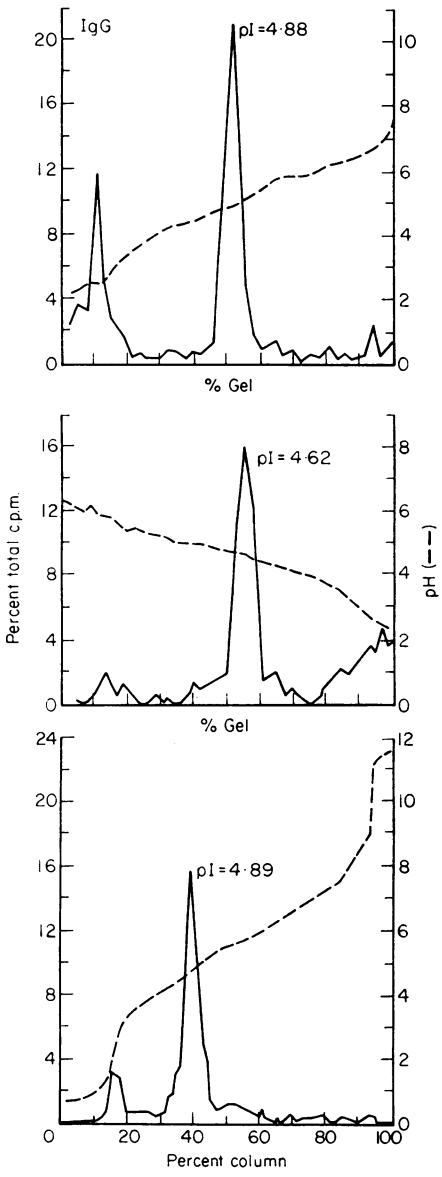

Figure 5. Isoelectric focusing profiles for heavy chains of IgM, IgA and IgG-Gil. Electrofocusing was performed over the pH range of 4–9 for 48 h at a constant voltage of 380. All solutions were 2 M in urea.

istic of the VKappa-ii variable region subgroup. This is the first instance in which this kappa chain type has been noted for rheumatoid factors.

The heavy chains of these antibodies were also isoelectric focused. The pI obtained were restricted in heterogeneity but dissimilar, being at pH 7·1, 7·0 and 6·7 for the IgG, IgA and IgM heavy chains respectively. Since the constant region of each heavy chain is dissimilar, no conclusion can be made concerning the similarity of the heavy chain variable region subgroups. These findings must await antigenic and structural analyses which are in progress.

The isoelectric points obtained for the light and heavy chains would seem to weaken our previous notion that the acidic pI produced by most intact rheumatoid factors may be due to substitutions of such amino acids as aspartic and glutamic acid in hypervariable segments of the light and heavy chains (Trieshmann *et al.*, 1975). When urea is utilized in the electrofocusing experiments, the isoelectric points of the anti-IgGs (and their light and heavy chains) increase dramatically. Thus, the inherent tertiary conformation of these proteins may be of importance for producing the acidic pI noted when most anti-IgGs are electrofocused in solutions which do not contain urea.

This is also the first note of the occurrence of monoclonal rheumatoid factors in the serum of patients with Felty's syndrome. The elevated levels and unusual high frequency of occurrence of serum anti-IgGs in this syndrome have been previously emphasized. Ruderman, Miller & Pinals (1968), in their retrospective study of twenty seven patients, recorded that anti-IgGs were present in 21/21 patients whose sera were tested. Similarly, Barnes, Turnbull & Vernon-Roberts (1971) noted anti-IgG activity in 19/21 sera, usually in high titre. Studies to be reported from this laboratory indicate that sera from three of seven patients with Felty's syndrome that gave titres of anti-IgG activity of greater than 1:5120 by standard tube latex agglutination assay contained monoclonal IgM and IgG, or IgM and IgA rheumatoid factors. Thus, the finding of triclonal gammopathies in this rheumatoid arthritis variant may not be uncommon.

These sera and their constituent anti-IgGs offer a means to study the autoimmune response and the structural and antigenic relationships between autoantibodies of like specificity and different immunoglobulin class. These anti-IgGs are found during

the course of an autoimmune disease state and hence their assay may offer new insights into the nature of the autoantibody response in autoimmune diseases in particular and the human immune response in general.

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Mrs Sally A. Hart prepared the manuscript.

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