ISOELECTRIC FOCUSING OF PROTEOLYTIC FRAGMENTS FROM RABBIT ANTI-STREPTOCOCCAL ANTIBODIES OF RESTRICTED HETEROGENEITY

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1. Introduction

The isoelectric spectrum of antibody has proven to be a convenient phenotypic marker of single clones of antibody-forming cells [1]. However, the product of a single clone of myeloma-producing cells or single clones of antibody-producing cells might exhibit multiple band isoelectric focusing patterns [1, 2]. Multiple bands of antibodies to *p*-azobenzoate hapten corresponded to a single light-chain band but several heavy-chain bands [3]. Structural and serological data [4], suggested that a rabbit antibody specific to Group C streptococcal polysaccharide had a minimum of two heavy-chains associated with a single light-chain. To further define the regions of the molecule responsible for the multiple band pattern of anti-carbohydrate antibodies of restricted heterogeneity, we have undertaken isoelectric focusing analysis of their pepsin and papain fragments. This study shows that focused papain Fab fragments of restricted antibodies have a smaller number of dominant bands than the original product. The multiple band isoelectric focusing pattern of restricted antibodies appears to be essentially due to the heterogeneity of Fc fragments and to the hinge region present between Fab and Fc.

2. Materials and methods

Antibodies to streptococcal group C carbohydrates (strain C74) were obtained from immune sera of rabbits which underwent 8 weeks of immunizations with whole bacteria according to the schedule of injections described [5]. The preparation of vaccine, the isolation of group-specific carbohydrate and the purification of antibody on immunoabsorbent were made as previously described [5, 6].

Papain fragments Fab and Fc were prepared according to Porter [7]. Pepsin digestion of antibodies was performed at pH 4.5, at 37°C for 16 hr [8]. Peptic digest was applied to a Sephadex G-200 column (1.5 × 150 cm); $F(ab')_2$ were in peak 1 and peak 3 contained Fc-like fragments.

Isoelectric focusing in thin layers of acrylamide gel was performed following published procedure [9]. Ampholine carrier ampholytes were of pH range 3–10.

3. Results

The striking fact which emerges from isoelectric spectra of anticarbohydrate antibodies and their papain fragments is the reduction of the number of dominant bands for Fab compared to the undigested material. This is apparent in each antibody preparation shown in fig. 1. Antibody 1689 composed of 5 dominant bands and several minor products yielded 2 major Fab fragments. Partially restricted antibody isolated from rabbit 1264 gave 3 major Fab fragments. One major component is seen in the acid pH range after papain action on antibody 1685. This phenomenon is not apparent in the case of Fab fragments obtained from normal IgG as both products are heterogenous. Papain Fc of normal IgG exhibits however 4–6 major bands as can be seen in the basic pH region of the ampholine gradient (fig. 1). Independently of the degree of restriction, antibody Fc fragments present also several bands comparable to those obtained from normal immuno-globulin.

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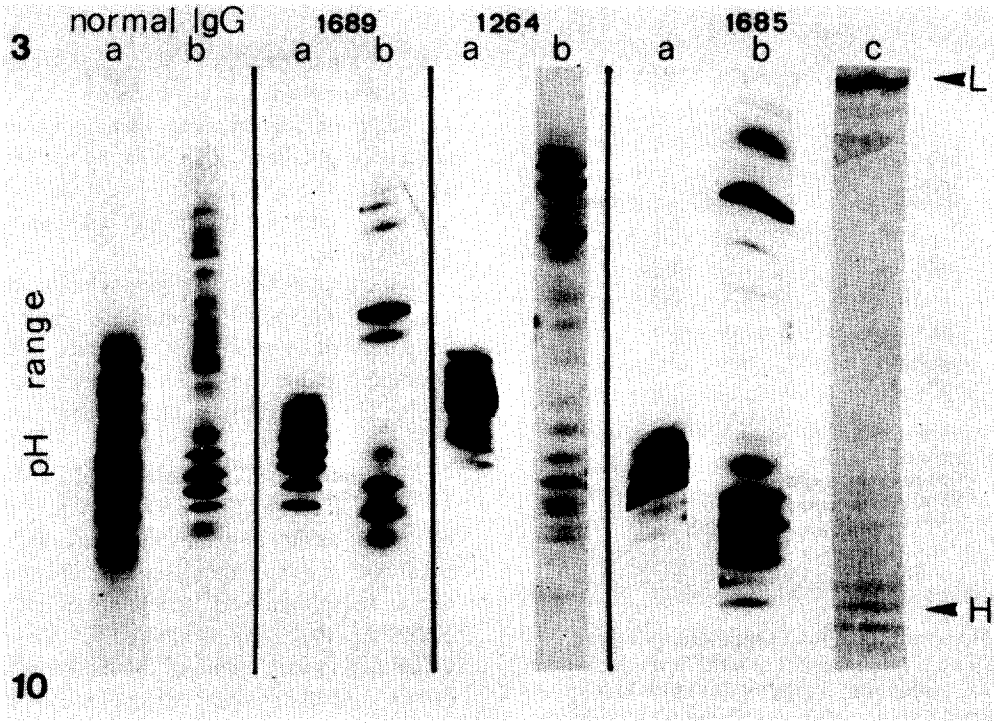

Fig. 1. Isoelectric spectra of rabbit non-specific IgG and three samples of antibodies of restricted heterogeneity specific for group C streptococcal carbohydrates (a). Total papain digest of homologous immunoglobulin and antibody samples (b). Fab fragments are located in the acid pH region of the gradient (top), Fc fragments are seen in the basic pH range (bottom). Isoelectric focusing of light- and heavy-chains of completely reduced and alkylated antibody sample 1685 (c).

That the isoelectric heterogeneity observed for antibody 1685 can be attributed to the heavy-chain and not to the light-chain was confirmed on the completely reduced and alkylated material. Isoelectric focusing shows that the antibody possess one dominant light-chain band and 3 heavy-chain bands (fig. 1).

To further show the contribution of various regions of the molecule to the isoelectric heterogeneity of antibodies, several preparations were digested with pepsin. Isoelectric spectra of purified pepsin $F(ab')_2$ and Fab from antibody 1685 and 1689 are compared in fig. 2. It can be seen that $F(ab')_2$ fragments exhibit a comparable number of bands to those present in the intact antibody. Thus the pattern of papain Fab fragments presents not only more simplicity than the intact antibody but is also less complex than the homologous $F(ab')_2$ pattern. It should be noted that pepsin Fc subfragments [10] localized in the basic region of the pH 3–10 gradient show heterogeneous spectra as each antibody digest focused has 4 bands which correspond to this portion of the molecule (fig. 2).

Because the divalent fragment $F(ab')_2$ differs from the papain fragment by the hinge region which is part of the heavy-chain in the peptic fragment but is absent in the papain Fab [11], it was suspected that the hinge peptide may play a role in the heterogeneity of $F(ab')_2$ fragments. Therefore digestion with papain was performed on the pepsin fragment $F(ab')_2$ and the product was submitted to isoelectric focusing. The result obtained with antibody 1689 confirmed the above prediction. Digestion of $F(ab')_2$ with papain yielded Fab fragments more restricted than the starting product. Also, the patterns of Fab obtained by conversion of pepsin fragments or by digestion of the intact antibody by papain were identical.

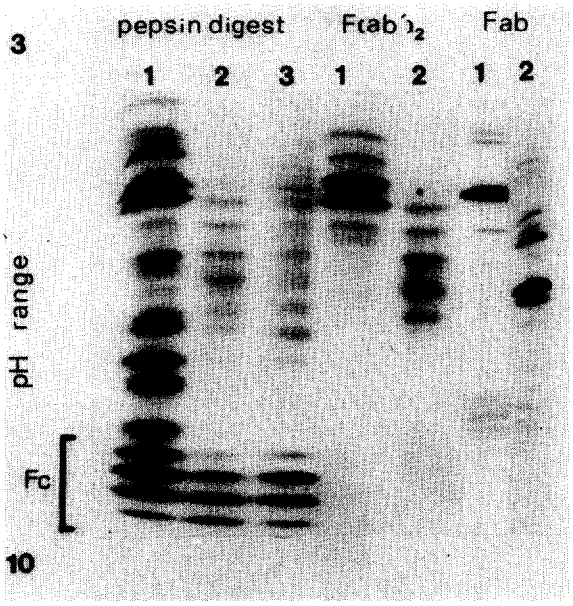

Fig. 2. Isoelectric spectra of total pepsin digest, isolated $F(ab')_2$ and papain Fab fragments of rabbit anti-cabohydrate antibodies. (1) sample 1685, (2) 1689, (3) 1675. $F(ab')_2$ were isolated after gel filtration of the pepsin digest on Sephadex G-200. Papain Fab were recovered from the supernatent solution after crystalization of Fc fragments.

4. Discussion

Papain Fab fragments from antibodies of restricted heterogeneity exhibit isoelectric spectra less complex than the intact antibodies. However, pepsin-derived $(Fab')_2$ fragments and the undigested product possessed a comparable number of dominant bands. It may be concluded that the heterogeneity of focused antibodies is partially due to the hinge region which is present in the peptic frament $(Fab')_2$, but is absent in the papain fragment Fab [11]. The reduced complexity of the pattern of Fab fragments obtained by digestion of pepsin $F(ab')_2$ by papain supports this conclusion. On the other hand, both papain Fc and pepsin Fc-like fragments of restricted antibodies are generally composed of 4–6 dominant isoelectric components. The multiple banding pattern observed, was previously mentioned for Fc and its subfragments of human heavy-chains subjected to gel electrophoresis, even if the fragments were obtained from purified myeloma IgG [12]. The regular banding of Fc and Fc-like fragments, probably due to uniform charge differences, suggests that this portion of the molecule exhibits minor degrees of structural variations [13]. The present work also confirms previous reports [3, 4] that the isoelectric heterogeneity observed for restricted antibodies is a property of the heavy-chains and not of the light-chains as multiple bands seen in antibody 1685 yielded a single light-chain but several heavy-chains.

The question which arises is whether the heterogeneity of the hinge region and Fc fragments is genetically defined by amino acid sequence variations or whether it is due to post biosynthetic charge alterations [2] or variations in carbohydrate content [11, 14].

The findings of Askonas et al. [1] are of great importance, because of the possibility that the product of a single antibody-producing clone might exhibit isoelectric focusing heterogeneity. This has been justified as an postsynthetic charge alteration of immunoglobulin probably by amide loss [2]. The hinge region which is singularly exposed to enzyme attack will also be the most likely target for in vivo alterations. Variations in amino sugar content of the hinge peptide were reported. The single residue of galactosamine linked to threonine – 225 is present on only 35% of the rabbit heavy-chains [11]. This site however is also subject to amino acid substitutions (threonine to methionine), in relation with allotypic specificities, A 11 and A 12 [15]. Finally, characterization of different glycopeptides derived from Fc region of heavy-chain point to the existence of rabbit IgG subclass [16].

The conclusion of this study is that variations in the hinge region which link Fd and Fc portions of heavy-chains and differences in Fc regions, contribute to the diversity of rabbit antibodies of restricted heterogeneity which may possess identical or very similar Fab regions. Whatever the origin of the heterogeneity of the constant region of H chain may be, isoelectric spectra of papain Fab fragments of restricted antibodies reflects better than the intact molecule the 'monoclonal' nature of the combining region of antibodies and should preferentially be used as a phenotypic marker of antibody-producing cells.

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References

[1] Askonas, B.A., Williamson, A.R. and Wright, B.E.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1398–1403.
[2] Awdeh, Z.L., Williamson, A.R. and Askonas, B.A. (1970) Biochem. J. 116, 241–248.
[3] Hoffman, D.R., Grossberg, A.L. and Pressman, D. (1971) J. Immunol. 107, 325–330.
[4] Waterfield, M.D., Prahl, J.W., Hood, L.E., Kindt, T.J. and Krause, R.M. (1972) Nature New Biol. 240, 215–217.
[5] Osterland, C.K., Miller, E.J., Karakawa, W.W. and Krause, R.M. (1966) J. Exptl. Med. 123, 599–614.
[6] Eichmann, K. and Greenblatt, J. (1971) J. Exptl. Med. 133, 424–441.
[7] Porter, R.R. (1959) Biochem. J. 73, 119–126.
[8] Mandy, W.J., Rivers, M.M. and Nisonoff, A. (1961) J. Biol. Chem. 236, 3221–3226.
[9] Awdeh, Z.L., Williamson, A.R. and Askonas, B.A. (1968) Natura 219, 66–67.
[10] Utsumi, S. and Karush (1965) Biochemistry 4, 1766–1779.
[11] Smyth, D.S. and Utsumi, S. (1967) Nature 216, 332–335.
[12] Heimer, R. and Schnoll, S.H. (1968) J. Immunol. 100, 231–237.
[13] Frommel, D. and Hong, R. (1970) Biochim. Biophys. Acta 200, 113–124.
[14] Kornfeld, R., Keller, J., Baenziger, J. and Kornfeld, S. (1971) J. Biol. Chem. 246, 3259–3267.
[15] Prahl, J.W., Mandy, W.J. and Todd, C.W. (1969) Biochemistry 8, 4935–4940.
[16] Florent, G., De Vries, G.M. and Hamers, R. (1973) Immunochemistry 10, 425–430.