LIMITATIONS IN THE APPLICABILITY OF THE COMPLEMENT-FIXATION TEST FOR THE ESTIMATION OF SPECIFICITY OF HISTONE ANTISERA

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1. Introduction

Among the different histone classes, the lysine-rich histone class reveals the greatest intra-specific variability [1,2]. This class contains a number of histone H1 variants. A remarkable lysine-rich histone variant is histone H5, that has been described as erythrocyte specific in a variety of species [3–5] among which *Xenopus* [6].

In order to determine the developmental stage in which H5 appears and to analyse in detail in which cells this occurs, we set up an indirect immunofluorescent technique on tissue sections from several developmental stages, using antibodies elicited against H1 and H5 from *Xenopus*, respectively. These antibodies were specific as determined by a complement fixation assay and by an in situ immune peroxidase assay with histones fractionated on a polyacrylamide gel as shown before [6].

Unexpectedly, we found in tissue sections from *Xenopus laevis* tadpoles, that anti-H5 serum reacted, not only with erythrocyte nuclei, but also with nuclei from other cell types, that are not considered to differentiate into erythrocytes (e.g. myotomes) [7]. This observation could be of potential importance for considerations on developmental mechanisms of tissue differentiation. However, a trivial explanation that would invalidate this finding would be a cross-reaction of this anti-H5 serum with histone H1, because of the expected high degree of homology between H1 and H5 (cf. [8]).

Thus, in spite of the observed specificity in the complement-fixation assay, the finding of H5 in other than erythropoietic cells casted doubt on the adequacy of the previous tests and on the specificity of the antiserum. Therefore, we have extended the above mentioned tests, with a modified enzyme-linked immunoassay and with a radioimmunoassay, in which we determined whether H1 was able to compete with H5 for binding to anti-H5. While the specificity of the antiserum both in the complement-fixation assay and in the in situ immune peroxidase test could be confirmed [6], a crossreactivity with histone H1 was found in the enzyme-linked immunoassay and the radioimmunoassay.

We conclude that specificity in the complement-fixation test (an assay on specificity, that is widely used in the field of histone immunology [9]) does not permit unambiguous conclusions on the specificity of an antiserum.

2. Methods and materials

2.1. *Isolation and further fractionation of lysine-rich histones from* Xenopus *erythrocytes*

Blood was obtained from anaesthetised animals (*Xenopus laevis laevis* (Daudin)). Anaesthetisation was performed by immersion of the animals for 15 min in a 0.1% solution of the anaesthetic MS 222 (Sandoz). The peritoneal and pericardial cavities were opened, the arterial pole of the heart was cut and blood was collected with a needleless syringe, while pumping an isotonic solution [10] into the liver.

The isolation of nuclei and chromatin was essentially as described previously [6], with some minor

Abbreviations: IgG, immunoglobin G; PBS, 10 mM sodium phosphate, 150 mM NaCl (pH 7.2)

changes in the solutions to prevent occasionally occurring degradation. Erythrocytes were homogenised in 2.4 M sucrose, 3 mM $MgCl_2$, 50 mM $NaHSO_3$, 0.5% (v/v) Triton X-100, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride and 5 mM Tris–HCl (pH 7.5). Washing was twice with 20 mM EDTA, 50 mM $NaHSO_3$, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride and 10 mM Tris–HCl (pH 7.5) and twice with 10 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride.

Purification and fractionation of lysine-rich histones by ion-exchange chromatography was as described [6], except that chromatography was performed on Bio-Rex 70 (Bio-Rad, 200–400 mesh) [11] instead of Amberlite CG-50 II. Relevant fractions were analysed on acid-urea-Triton X-100 gels [12].

2.2. *Immunological procedures*

Immunization of rabbits with a complex of H5 and yeast RNA was performed according to Stollar and Ward [13].

Published procedures were used for the quantitative microcomplement-fixation reactions [6,13], for the radioimmunoassay [14] and for the test for specificity on gel sections of polyacrylamide gels in which histones were separated [15].

The enzyme-linked immunoassay was performed with polyvinylchloride discs (diameter: 5 mm). They were coated with antigen by an incubation with a solution of 100 μg H1A or H5 per ml for 2 h, at room temperature, followed by three washes with PBS and post-coated with 10% normal goat serum. Reaction with rabbit serum was with a 1 : 30 solution for 30 min at room temperature and 16 h at 4°C. After 3 washes with PBS, a second incubation with goat anti-rabbit IgG, covalently coupled to peroxidase, for 2 h at room temperature and again 3 washes with PBS, the discs were incubated with diaminobenzidine and H_2O_2 for 10 min [15]. If peroxidase was bound to the discs, a brown insoluble reaction product was found.

3. Results and discussion

Faced with the interesting possibility that nuclei from non-erythropoietic embryonic tissue could contain histone H5, we felt it absolutely necessary to apply more stringent criteria to exclude a possible aspecificity of the anti-H5 serum.

Since we had found that the anti-H5 serum reacted only with the nuclei of cells, not with the cytoplasm and, moreover, that pre-immune serum did not react at all (data not shown), we judged the immunofluorescence technique, as such, reliable. Therefore, we submitted the anti-H5 serum to a variety of tests on specificity, covering a broad range of sensitivities (incubations in liquid phase vs. solid phase assays), which led to different specificities in different tests.

3.1. *Purity of the antigens*

To permit comparison of the different specificity tests, all experiments were performed with one batch of purified antigen H1A and H5. For that purpose lysine-rich histones were fractionated on a Bio-Rex column (fig.1A), fractions were pooled as indicated and subjected to electrophoresis on an acid-urea-Triton gel (fig.1B).

Chromatography on Bio-Rex resulted into two H5 peaks (peaks 4 and 5) instead of one as was found after chromatography on Amberlite [6]. Although we do not have an explanation for this phenomenon as yet, we consider them both as H5 fractions, since they were isolated from erythrocyte nuclei according to a standard procedure for histones [6] and since they both have the same charge and MW in three different electrophoretic systems [12] as the apparently homogeneous H5 histone fraction described earlier [6] (data not shown). In further experiments peak 5 was used since it does not show contamination with H1 (fig.1B), for the same reason H1A (peak 2) was used to test its capability to react with anti-H5 serum.

3.2. *Specificity tests using both antigen and antibody in liquid phase*

Complement fixation is the most widely used technique for assaying specificity of histone antibodies (cf. [9]) both due to its sensitivity and its simplicity. Fig.2 shows that, H5 does induce complement fixation in a concentration range of 0.1–0.4 μg per ml, using an anti-H5 dilution of 1 : 15 000, while H1A, in the same concentration range as H5, does not react at all. So in complement fixing activity the serum appeared to be specific in agreement with our previous results [6].

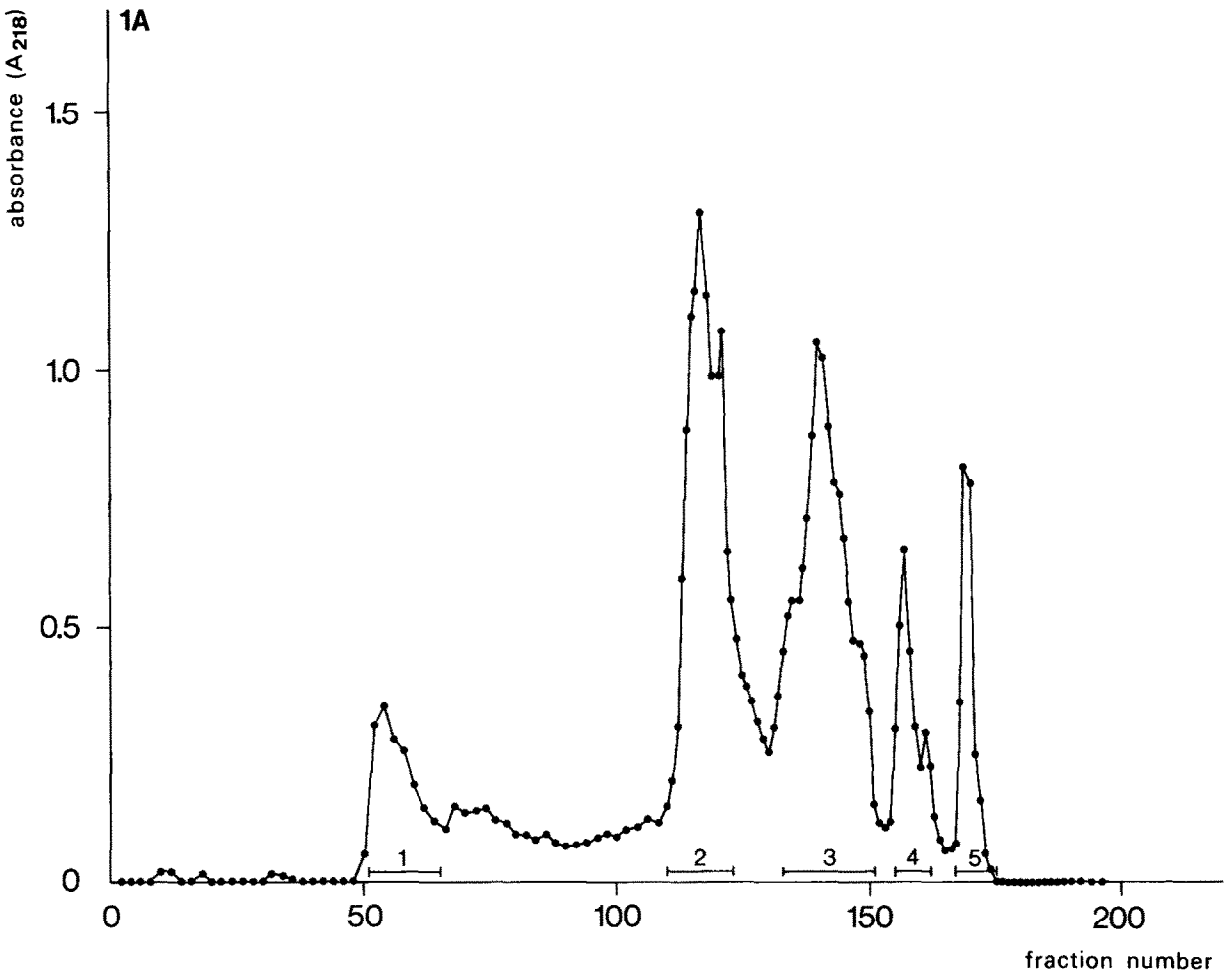

Fig.1(A) Fractionation of lysine-rich histones from *Xenopus* on a Bio-Rex 70 column (1.5 × 100 cm), using a 400 ml gradient of 8–15% guanidinium chloride and a flow rate of 4 ml/h. The fraction size was 2 ml (each second fraction is given in the figure) and fractions were pooled as indicated by bars.

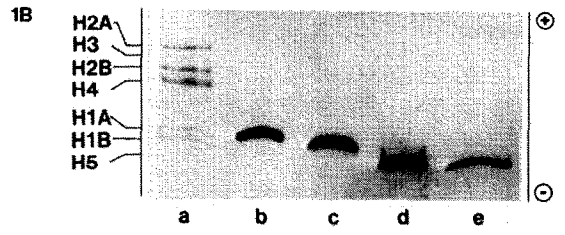

Fig.1(B) Acid-urea-Triton X-100 polyacrylamide gel electrophoresis [12] of some fractions pooled as indicated in fig.1A. Each lane contained 20 μg protein. Lane a: marker preparation of total histones; lane b: peak 2; lane c: peak 3; lane d: peak 4; lane e: peak 5.

Fig.2. Complement fixation of lysine-rich histones with antiserum against H5. Final serum dilution 1 : 15 000. •——•, reaction with H5; ○——○, reaction with H1A. Values given are the means of duplicate determinations. →

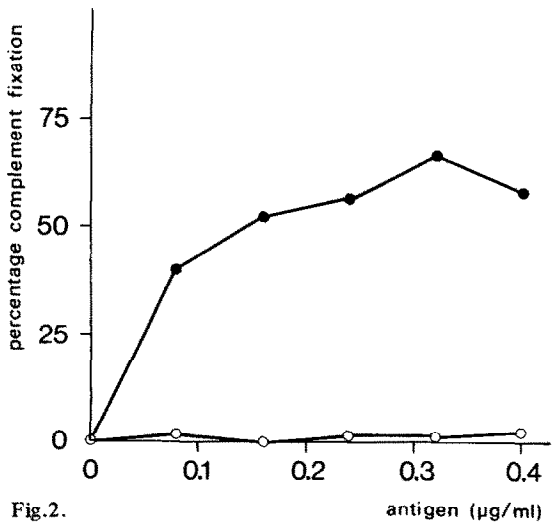

Fig.2.

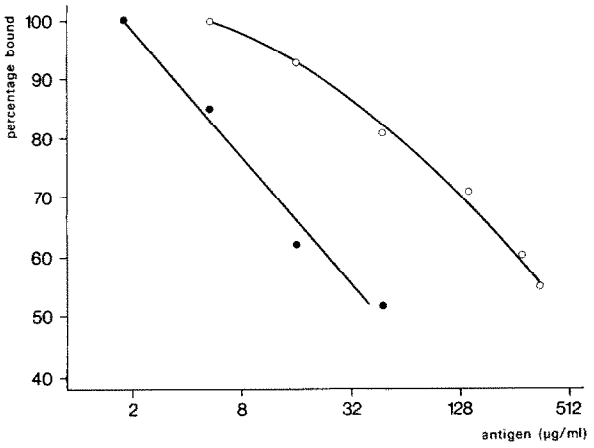

Fig.3. The effect of varying concentrations of unlabeled H1A or H5 on the binding of iodinated H5 to antiserum against H5. Final serum dilution 1 : 25. •——•, H5; ○——○, H1A. Radioimmunoassay was performed as described in ref. 14 with a minor modification: unlabeled histones were used without preincubation with detergent. In all cases a blank containing preimmune serum instead of antiserum was subtracted. The percentage binding obtained in the presence of 2 µg/ml of unlabeled ligand (15% of the input) was arbitrarily set at 100%. The input of ^{125}I-H5 was ~1 ng; i.e. 2500 cpm. Values given are the means of duplicate determinations.

However, this result contrasted with that obtained from the radioimmunoassay. As shown in fig.3, H1A competes effectively with ^{125}I-H5 for binding to anti-H5; albeit, 10–17 times more H1A than H5 is required to produce the same competition with ^{125}I-H5. This suggests a cross-reactivity of the anti-H5 serum with H1A of about 10–17%, depending on the concentration of antigen used, at least at the anti-H5 serum dilution used (1 : 25). This low serum dilution is compatible with that used in the chicken H5 radioimmunoassay, where an 1 : 8 serum dilution was used [16]. This is probably necessary because of damage of the iodinated protein. Taking into account that the ratio of antigen to antibody is about equal in both tests, and that the concentration of antigen and antibody is much higher in the radioimmunoassay than in the complement fixation tests an obvious explanation could be that the anti-H5 serum contains antibodies with low affinity for H1. These antibodies, therefore, could not be detected in the complement fixation test, since at the high dilutions used in this test, the equilibrium of the antigen antibody reaction is shifted to the dissociated state. Higher H1 concentrations could not be used in the complement fixation test since higher concentrations of antigen, in the absence of antibody, resulted in a considerable complement fixation.

To get better insight into the extent of the cross-reactivity we set up two solid phase techniques in which the antigen was bound, since these can be expected to reflect more the situation in tissue sections.

3.3. *Solid phase assays*

First we repeated the test described previously [6] involving an immunohistochemical reaction in gel sections from a polyacrylamide slab gel (presented in fig.1B). The gel sections were made 50 µm thick, containing about 250 ng protein, lengthwise and parallel to the gel surface, in a cryostat-microtome. They were incubated consecutively with anti-H5 or preimmune serum, goat anti-rabbit IgG covalently coupled to peroxidase, and diaminobenzidine and hydrogen peroxide as described [15]. Fig.4 shows that the anti-H5 serum is specific in this test: reaction products could only be detected with H5 and preimmune serum does not react with any of the antigens (cf. [6]) (data not shown).

In the second test applied, purified antigen (H1A or H5) was coated on PVC discs (about 20 ng per disc, as determined by binding of iodinated protein) and incubation with antisera was performed as described above for the gel section test. In this test

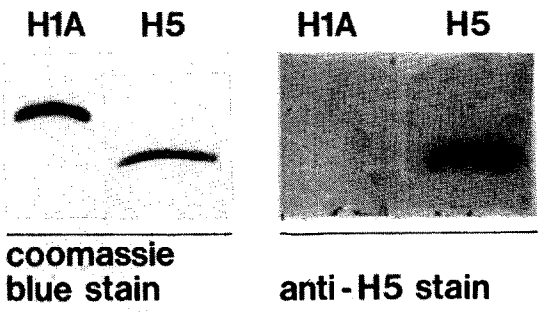

Fig.4. Reaction of the anti-H5 serum with H1A and H5 in polyacrylamide gel sections. Lanes b and e of fig.1B, containing H1A and H5 respectively, were sectioned in a number of 50 µm thick coupes as described [15]. Sections of both lanes were stained with either Coomassie blue to indicate the position of the protein (left panel), or incubated with anti-H5 serum (right panel), as described [6,15]. The first antibody reaction was with a 1 : 30 diluted rabbit antiserum, 30 min at room temperature, 16 h at 4°C; the second antibody reaction was for 2 h at room temperature with a 1 : 30 diluted goat anti-rabbit IgG-peroxidase (Miles).

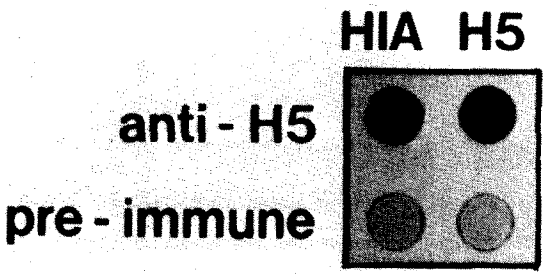

Fig.5. Specificity test of the anti-H5 serum using a modified enzyme-linked immunoassay. Polyvinylchloride discs were coated with H1A or H5, left and right vertical row, respectively and incubated with anti-H5 or preimmune serum, upper and under horizontal row, respectively. Incubations were as described in Section 2.

a slight crossreactivity of the anti-H5 serum with H1A could be demonstrated (fig.5), preimmune serum did not react. The discrepancy between these two solid phase assays cannot easily be explained since, although the antibody dilution used is in both tests in the same range the antigen concentration is about 10 times higher in the gel section test. An explanation could be that the gel section test is not sensitive enough to pick up the 10% crossreactivity with H1A, either due to loss of antigenic determinants in the acid-urea-Triton X-100 gel electrophoresis procedure, or due to inaccessibility of the antigen in the gel.

3.4. *Concluding remarks*

The results presented suggest that the anti-H5 serum used contains a slight crossreactivity with H1A that remained undetected in certain tests. The complement fixation test is incapable to detect this cross-reacting activity. Therefore, these results obviously impose severe limitations to its applicability both to test specificity and to test immunological distances.

This crossreactivity is more likely to be due to the presumed sequence homology between H1 and H5 of *Xenopus* (still up to 40% between trout H1 and goose H5 [8]), than to impurity of the antigen used for immunisation (see fig.1B). The fact that all attempts to purify the sera elicited against H5 by repeated treatment with purified H1, coupled to Sepharose beads, failed, supports the former explanation, too.

To circumvent laborious procedures for purifying antisera, we set up the hybridoma technology as designed by Köhler and Milstein [17] to obtain antibodies, specific for a single lysine-rich histone variant. Recently we have succeeded in the isolation of a clone producing IgG specific for H1A [18].

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References

[1] Bustin, M. and Cole, R. D. (1968) J. Biol. Chem. 243, 4500–4505.
[2] Kinkade, J. M. (1969) J. Biol. Chem. 344, 3375–3386.
[3] Neelin, J. M., Callahan, P. X., Lamb, D. C. and Murray, K. (1964) Can. J. Biochem. 42, 1743–1753.
[4] Vendrely, R. and Picaud, M. (1968) Exp. Cell Res. 49, 13–24.
[5] Edwards, L. J. and Hnilica, L. S. (1968) Experientia 24, 228–229.
[6] Destrée, O. H. J., Hoenders, H. J., Moorman, A. F. M. and Charles, R. (1979) Biochim. Biophys. Acta 577, 61–70.
[7] Hollyfield, J. G. (1966) Dev. Biol. 14, 461–480.
[8] Yaguchi, M., Roy, C. and Seligy, V. L. (1979) Biochem. Biophys. Res. Commun. 76, 1400–1406.
[9] Bustin, M. (1978) in: The Cell Nucleus (Bush, H. ed) vol. IV, pp. 195–238, Academic Press, New York.
[10] Stearn, R. N. and Kostellow, A. B. (1958) in: The Chemical basis of development (McElroy, W. D. and Glass, B. eds) pp. 448–457, The John Hopkins Press, Baltimore.
[11] Fambrough, D. M., Fujimura, F. and Bonner, J. (1968) Biochemistry 7, 575–585.
[12] Koster, J. G., Kasinsky, H. E. and Destrée, O. H. J. (1979) Cell Diff. 8, 93–104.
[13] Stollar, B. D. and Ward, M. (1970) J. Biol. Chem. 245, 1261–1266.
[14] Charles, R., De Graaf, A. and Moorman, A. F. M. (1980) Biochim. Biophys. Acta 629, 36–49.
[15] Van Raamsdonk, W., Pool, C. W. and Heyting, C. (1977) J. Immunol. Methods 17, 337–348.
[16] Goetz, G., Esmailzadeh, A. K. and Huang, P. C. (1978) Biochim. Biophys. Acta 517, 236–245.
[17] Köhler, G. and Milstein C. (1975) Nature 256, 495–497.
[18] Moorman, A. F. M., Amons, F., Charles, R., Cossé, A. A., De Graaf, A. and Roest, J. R. (1980) 2nd Int. Cong. on Cell Biology (Berlin).