BIOCHEMICAL CHARACTERIZATION OF MURINE BONE MARROW GP71

Nazario RUBIO*
Basic Research Program, NCI Frederick Cancer Research Center, Frederick, MD 21701, USA

Received 21 September 1981

1. Introduction

During the last years, increasing attention has been focused on the non-coordinate expression of murine leukemia virus envelope glycoproteins in the organs and serum from different inbred strains of mice [1–4]. Most of these proteins have been detected by using broadly reactive interspecies competition radioimmunoassays. The purification of the endogenous AKR-type virus gp71 [5] was used to develop an homologous type-specific assay against that protein. By using such an assay, an AKR gp71-like antigen has been detected in the bone marrow of all strains of inbred as well as wild mice tested [4]. These strains include high, low and non-virus strains in addition to NIH Swiss, a strain lacking a portion (<50%) of the viral genome [6]. That AKR-like gp71 is so highly conserved evolutionary in bone marrow cells and that this antigen can not be demonstrated in peripheral lymphoid organs, could indicate that this protein plays some role in a primitive stage of lymphoid development and differentiation. Lymphoid cell differentiation is currently one of the best understood. The aim of this work is to purify the AKR-like gp71 antigen from mouse bone marrow, to further establish its nature and compare it structurally with some viral proteins.

2. Materials and methods

AKR gp71, Rauscher gp71, Moloney gp71 and Balb/c (X) gp71 were purified as in [5]. Goat anti-Tween–ether-disrupted AKR MuLV and Moloney-MuLV antisera was obtained through the Resources and Logistics Program of the National Cancer Institute. Antiserum against AKR gp71 was prepared as in [5]. Viral and bone marrow proteins were labeled with $Na^{125}I$ according to [7].

Bone marrow cells from femurs and tibiae of Balb/c and NIH Swiss mice were homogenized in 1% Triton X-100, 1 M KCl, 0.1 M sodium phosphate buffer (pH 7) containing 1 μg/ml phenylmethylsulfonyl fluoride (PMSF). After centrifugation, the post-microsomal cell extracts were stored in liquid nitrogen. Protein concentrations were determined as in [8].

2.1. *Purification*

Homogenate (1 ml) from 25 bone marrows (45 mg protein) was applied to a 2 × 35 cm column of Sepharose 4B which was eluted at 4°C with TNE (0.01 M Tris–HCl, 0.1 M NaCl, 0.001 M EDTA (pH 7.5)) containing 1 μg PMSF/ml. Fractions (2 ml) were collected and monitored for absorbance at 280 nm. Each fraction was tested for competition in the homologous anti-AKR gp71–AKR gp71 cRIA. Fractions containing competing activity were pooled, dialyzed overnight against 0.01 M Tris–HCl, 0.001 M EDTA (pH 7.8) and applied to a Whatman DE-32 column pre-equilibrated with the same buffer. After washing, bound proteins were eluted with a linear gradient of KCl (0–0.5 M). Individual fractions were tested for protein and, after dialysis against TNE, assayed for AKR gp71 reactivity in cRIA.

For immunoaffinity chromatography 0.4 mg pure Moloney gp71 were coupled to 1 ml activated Sepharose 4B following the standard Pharmacia procedure. A high titer goat anti-Tween–ether-disrupted Moloney leukemia virus was used as a source of anti-group-specific antibodies. Heat-inactivated (56°C, 30 min) antiserum (1 ml) diluted 1:5 in PBS, was passed over the Moloney gp71 column at 5 ml/h. The column was washed with PBS until free of absorbance at 280 nm and the bound antibody eluted with 3 M KSCN. After dialysis against PBS the antibody prepa-

* Present address: 'Centro de Inmunología Experimental y Aplicada' INMAT, Bolivia, 25. Vigo, Spain

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

ration (3.6 mg) was concentrated by Diaflo P-30 ultrafiltration and checked for precipitating activity against ^{125}I-Moloney gp71. That antibody preparation was coupled as above to 1 ml activated Sepharose 4B and used for bone marrow gp71 immunoaffinity purification. The peak activity fractions of the DEAE-cellulose column were passed on the above column, which was pre-washed with TNE. After washing, the column was eluted with a gradient of trichloroacetic acid (0–2 M) pH 6.9 at 4°C. Following exhaustive dialysis against TNE, the eluted gp71 was concentrated by vacuum dialysis and labelled with ^{125}I.

This material was further purified by immunoprecipitation, essentially as in [9]. The labelled antigen was incubated with the appropriate amount of anti-AKR–MuLV antiserum or normal control serum and incubated for 1 h at 37°C and 4°C overnight. Then 100 μl of a 10% suspension of killed *Staphylococcus aureus* Cowan strain pre-washed with TNE + 0.1% Triton X-100 was added and the mixture incubated for 15 min at room temperature. The antigen–antibody–*Staphylococcus* complex was processed as in [10]. Briefly, after 3 washes with TNET, the complex was boiled in 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, 0.01 M sodium phosphate (pH 7) for 1 min, centrifuged and the supernatant subjected to SDS–polyacrylamide gel electrophoresis as in [11].

2.2. *Peptide mapping*

The peptide mapping was performed as follows: 1 mm slices from polyacrylamide gel electrophoresis containing the pure gp71 were pooled, washed overnight in 50% methanol, 10% acetic acid and digested overnight with 50 μg TPCK–trypsin (Worthington) in 0.1 M ammonium bicarbonate (pH 8). The buffer containing the peptides was lyophilized, rehydrated in the chromatographic eluent and spotted on cellulose thin-layer chromatography plates for two-dimensional peptide mapping according to the procedures in [12,13].

2.3. *Lectin affinity chromatography*

For lectin column affinity chromatography, 20 mg lectin from *Lens culinaris* (Boehringer Mannheim Biochemicals) were bound to 2 g activated Sepharose 4B in the presence of 0.1 M α-methyl-glucopyranoside. Concanavalin A–Sepharose was purchased from Pharmacia Fine Chemicals. Labelled samples in phosphate buffered saline (PBS) containing BSA (0.5 mg/ml) were added to 0.5 × 3 cm, siliconized columns at room temperature and then washed with the same buffer until cpm reached background levels. Bound protein was eluted by washing the columns with 0.2 M α-methyl-mannopyranoside.

3. Results

3.1. *Bone marrow AKR-MuLV gp71-like purification*

The Sepharose 4B column was used to remove nucleic acid and a part of the concomitant proteins. The results of a typical chromatography of a bone marrow homogenate sample is shown in fig.1. A first

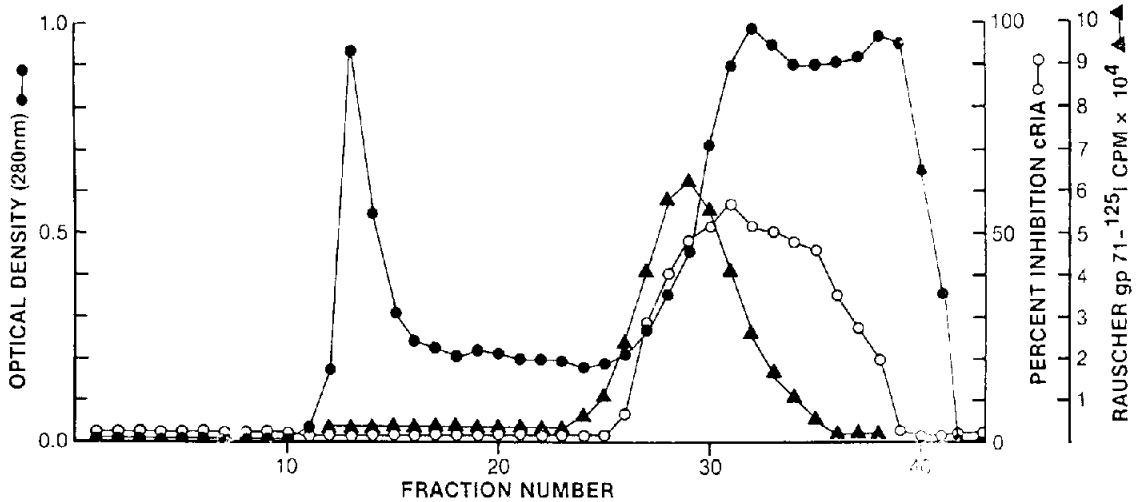

Fig.1. Sepharose 4B elution profile of murine bone marrow homogenate. The chromatography conditions were as in section 2.

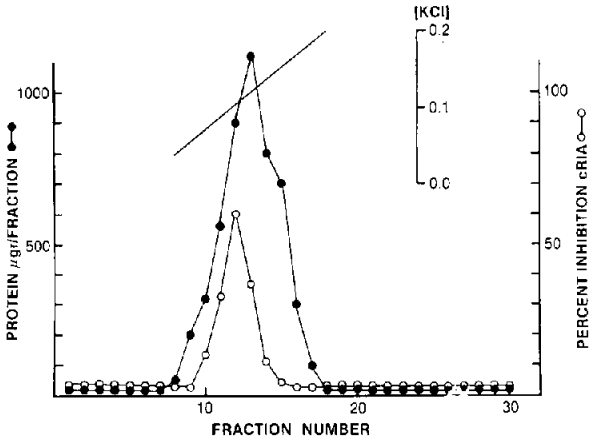

Fig.2. DEAE-cellulose chromatography of the active fractions from fig.1. After dialysis, the fractions were pooled and chromatographed on a DEAE-cellulose column as in section 2. KCl concentrations were determined by conductivity measurements.

peak of nucleic acid is excluded from the column, followed by a flat plateau of medium weight material and another two-shouldered peak. The bone marrow material that competes in the AKR gp71 homologous cRIA elutes as a broad peak in the first half of the last protein peak. The elution position of that material is roughly the same as that occupied by a sample of ^{125}I-Rauscher gp71. The broad shape of the tissue AKR gp71-like protein peak can be explained by some degradation of the material or by the experimental fact that glycoproteins are non-specifically retained in agarose columns. Fractions with the highest level of reactivity were pooled, dialyzed and applied to a DEAE-cellulose column. The elution profile obtained is illustrated in fig.2. One peak of competing activity is eluted from the column at 0.1 M

Table 1
Purification of AKR gp71 from mouse bone marrow

Purification step	Total protein (mg)	Purification (-fold)	Yield (%)
Crude homogenate	115	1	100
Sepharose 4B	28	4	95
DEAE-cellulose	6.3	18	36
Immunoaffinity	0.003	38.3	13

Mouse bone marrow gp71 was purified by a 3-step purification procedure according to section 2.2. Final yield was 13% with 38.3-times purification

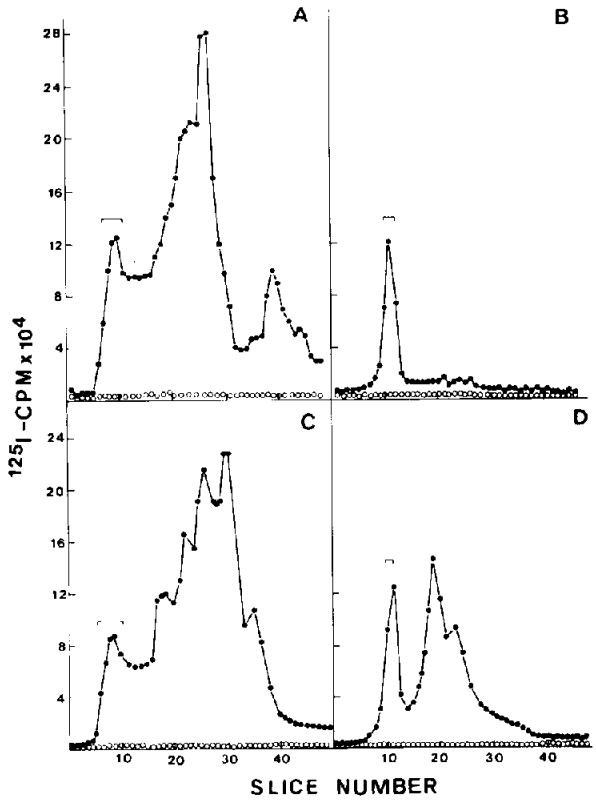

Fig.3. SDS–polyacrylamide gel electrophoresis of ^{125}I-labelled gp71 preparations purified by immunoprecipitation. Bars represent slices of the gels pooled for peptide mapping: (A) Balb/c bone marrow gp71; (B) AKR MuLV gp71; (C) NIH Swiss bone marrow gp71; (D) Balb (X) gp71.

KCl. This peak elutes slightly ahead of the major protein peak. Nevertheless, a considerable purification and concentration of the AKR gp71-like activity is obtained by using such a column. The 2 preceding steps were necessary to accomplish a satisfactory purification with the antibody affinity column. A high titer goat anti-Moloney MuLV antiserum was chosen as a source of group-specific antibodies against the AKR gp71-like bone marrow antigen, to prepare the immunoaffinity column. Previous competition RIA demonstrate that the bone marrow extracts compete in the group specific assay. The elution of the tissue antigen for such a column was ~30% efficient and the final yield of ~13% (table 1). The material eluted from the column was labelled with ^{125}I and immunoprecipitated with increasing 2-fold dilutions of rabbit anti-AKR gp71 or goat anti-AKR-MuLV. A binding plateau of 25% maximum precipitation was

reached with both antisera (not shown) indicating that only this percent of material is still able to bind antibody.

3.2. *Peptide mapping*

Both viral and bone marrow gp71 were further purified by immunoprecipitation. Of the counts used, ~28% were consistently recovered bound to the Staphylococci complex. After boiling, the supernatants were run on SDS–polyacrylamide gels. The profiles obtained were as illustrated in fig.3.

A discrete peak moving to the same zone in which viral gp71 is found. Nevertheless, a significant breakdown can be observed. The fragments seem to retain their immunological capacity since they are still able to bind antibody. Fig.4 shows the tryptic peptide maps of bone marrow, AKR-MuLV and Balb (X) gp71. A clear distinct pattern of spot distribution is observed in each case. The comparison of the bone marrow antigens with both viral products shows that the tissue gp71 resembles more the xenotropic than the AKR map. The NIH bone marrow protein looks like the xenotropic virus gp71 too. Thus, the antigenic relation is not accompanied by a clear structural similarity.

3.3. *Glycosylated nature of the bone marrow antigens*

Concanavalin A and *Lens culinaris* lectin have affinity for glucose and mannose containing glycoproteins. We tested that ability of both lectins covalently linked to Sepharose 4B to bind the immunoaffinity-purified bone marrow antigens as well as AKR MuLV gp71. The results of those affinity chromatography experiments are summarized in table 2.

Both NIH Swiss and Balb/c bone marrow preparations bind in a significant extent to the immobilized

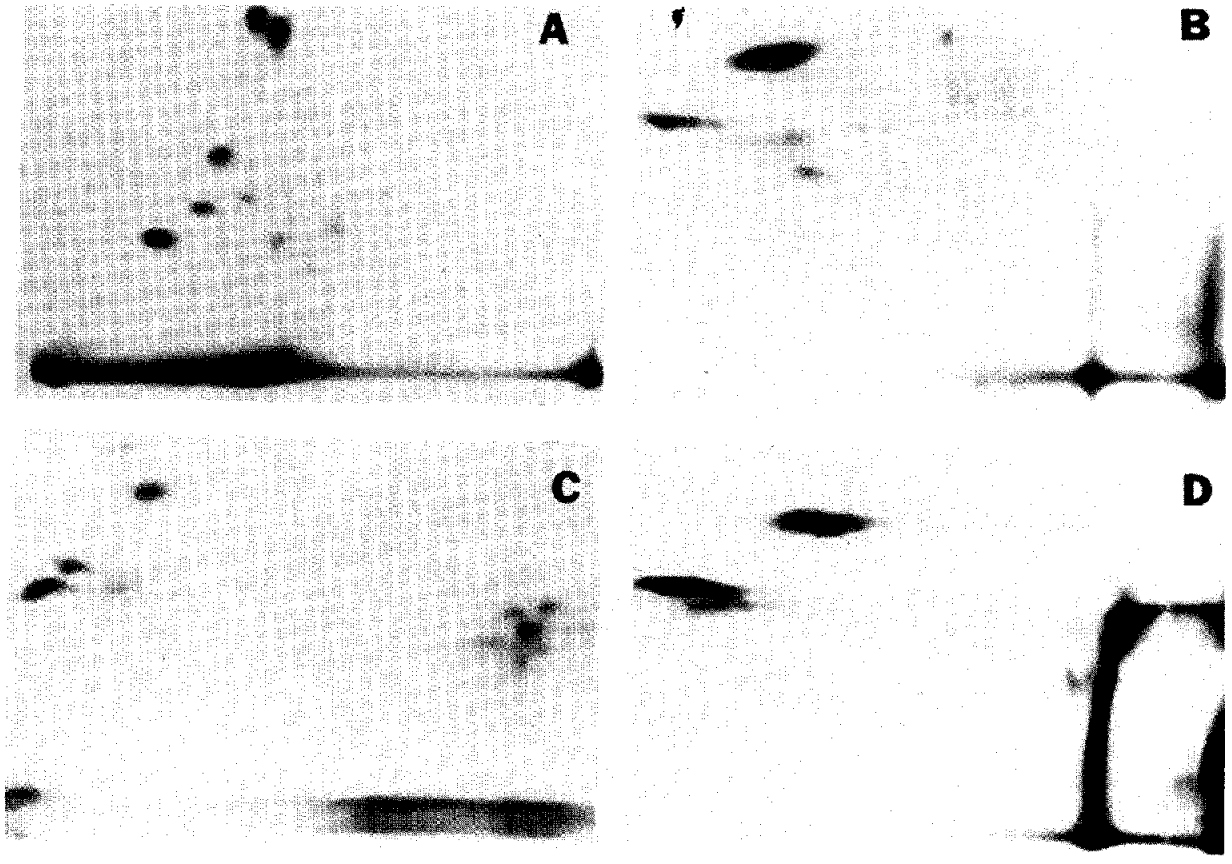

Fig.4. Comparison of the tyrosine-containing tryptic peptides of: (A) AKR MuLV gp71; (B) Balb (X) MuLV gp71; (C) Balb/c bone marrow; (D) NIH Swiss bone marrow gp71.

Table 2
Lectin affinity chromatography of bone marrow and AKR MuLV gp71

Chromatographed sample	Con A column (%)	Lentil lectin column (%)	Sepharose 4B column (%)
NIH bone marrow antigen	66	31	15
Balb/c bone marrow antigen	47	39	18
AKR MuLV gp71	92	80	21

Percent of binding to immobilized lectins and Sepharose matrix from 3 ^{125}I-labelled samples

lectins. Concanavalin A seems to have higher affinity than the lentil lectin for both antigens, but when we eluted the bound protein with methyl mannoside, only 22% is recovered. Elution from the lentil lectin column is almost twice as efficient (43%). Despite the addition of BSA to the chromatography buffer, ~15% of the input cpm binds non-specifically to a Sepharose 4B column. The results of the chromatography of a sample of AKR gp71 is shown for comparison. 92% and 80% of the input cpm bind to the lectins and some 21% non-specific binding is found. This particular preparation of gp71 was 95% precipitable by the antiserum. Keeping in mind that our bone marrow preparations are only 25% precipitable, we conclude that this material is glycosylated in nature. The elution behavior from the Sepharose column (fig.1) and the migration in the SDS–polyacrylamide gels in the same position as the viral gp71 control (fig.3), allow us to conclude that the bone marrow antigen is also a glycoprotein of 70 000–71 000 M_r.

4. Discussion

The AKR gp71-like antigen accounts for 180 and 140 ng/mg total bone marrow protein in Balb/c and NIH Swiss mice, respectively. The mean values for seric gp71 in 2-month-old animals of the same strains are 260 and 240 ng/mg [4]; those antigens are highly tissue-specific, the xenotropic gp71 being detected only in serum [4]. The bone marrow is the main source of precursors of lymphoid as well as hemopoietic cells. The fact that no AKR-like antigen can be detected in peripheral lymphoid organs could indicate that it is a host product playing some role in the early stages of lymphoid or erythroid cell differentiation. A direct comparison of the primary structure of the bone marrow product with viral envelope proteins can help to answer the question if this antigen is more 'virus-like' than 'host-like'.

Here, we report the comparison of the tyrosine-containing peptide maps of the purified bone marrow product with those of the gp71 of 2 viruses known to be harbored by NIH Swiss and Balb/c mice. In fact, the Balb/c strain contains genetic information coding for at least 3 distinguishable type-C oncornavirus [14]. NIH Swiss contains information for type II and III xenotropic virus production [3] and partial information for type I virus [6]. The patterns of spot distribution in the bone marrow gp71 peptide maps seem to indicate some similarity with the Balb (X) gp71 rather than with the AKR glycoprotein. Both tissue products seem to be distinct from the purified viral proteins, suggesting they are more 'host-like' complex products bearing some antigenic determinants from the envelope viral product of endogenous type I MuLV. This complex cellular product could be the result of a recombinant event.

Acknowledgement

Research sponsored by the National Cancer Institute under contract no. N01-CO-25423 with Litton Bionectics, Inc.

References

[1] Strand, M. and August, J. T. (1976) Virology 75, 130–144.
[2] Lerner, F. A., Wilson, C. B., Del Villano, B. C., McConahey, P. J. and Dixon, F. J. (1976) J. Exp. Med. 143, 151–166.
[3] Hino, S., Stephenson, J. R. and Aaronson, S. A. (1976) J. Virol. 18, 933–941.
[4] McClintock, P. R., Ihle, J. N. and Joseph, D. R. (1977) J. Exp. Med. 146, 422–434.
[5] Ihle, J. N., Denny, T. P. and Bolognesi, D. P. (1976) J. Virol. 17, 727–736.
[6] Chattopadhyay, S. D., Lowry, D. R., Teich, N. M., Levine, A. S. and Rowe, W. P. (1974) Proc. Natl. Acad. Sci. USA 71, 167–171.

[7] Greenwood, F. C., Hunter, W. M. and Glover, J. W. (1963) Biochem. J. 89, 114–123.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Kennel, S. J. (1976) J. Biol. Chem. 251, 6197–6204.
[10] Cullen, S. E. and Schwartz, B. D. (1976) J. Immunol. 117, 136–142.
[11] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[12] Gibson, W. (1974) Virology 62, 319–336.
[13] Morrison, T. G. and Lodish, H. F. (1975) J. Biol. Chem. 250, 6955–6962.
[14] Stephenson, J. R., Reynolds, R. K., Tronick, S. R. and Aaronson, S. A. (1975) Virology 67, 404–414.