PURIFICATION OF SOLUBLE MURINE TRANSPLANTATION ANTIGENS BY ISOELECTRIC FOCUSING

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Hydrosoluble transplantation antigens were prepared from membranes and microsomes of C_3H mice (BP8 tumoral cells) and purified by isoelectric focusing. A biologically active fraction which seems homogenous by acrylamide gel electrophoresis was characterized: it specifically inhibits anti C_3H hemagglutinin antibodies and provokes a highly significant prolongation of skin graft.

1. Introduction

Several research groups have shown independently during the last few years the association of histocompatibility-2 antigens to a lipoproteic fraction obtained from microsomes or from cellular membranes. The solubilisation and purification of this material was later tried by different methods [1–5].

Our group described the preparation of hydrosoluble fractions from normal and BP8 tumoral murine (C_3H) cells (microsomes and membranes) either by treatment with a mixture of solvents [6] or by autodigestion [7]. These fractions were biologically active: *in vivo*, they provoked the formation of cytotoxic and hemagglutinin antibodies and caused an accelerated rejection or prolongation of skin grafts; *in vitro*, they specifically inhibited anti C_3H cytotoxic and hemagglutinin antibodies. By chromatography on DEAE-cellulose, purified hydrosoluble biologically active fractions were obtained; it was possible for these to prolong the skin grafts. However, acrylamide gel electrophoresis of the active fractions after DEAE-cellulose chromatography always indicated a high complexity. For this reason we tried to purify, by isoelectric focusing, the soluble material obtained by autodigestion from BP8 murine (C_3H) tumoral cells.

2. Materials and methods

The antigenic material was prepared from lots of 80 three to five months old C_3H mice. $C_{57}Bl/6$ mice (H-2b) were used for the preparation of the antisera necessary for the *in vitro* test and also as receptors of the allogenic skin grafts. The test of inhibition of hemagglutination was achieved by the procedure of Gorer [8] slightly modified in our laboratory.

The unit of specific activity is represented by the minimal concentration of the tested product in $\mu g/ml$ able to inhibit the action of an equal volume of hemagglutinating antiserum, the dilution of antiserum chosen being the last but one giving a net positive reaction.

For the skin grafts, the fraction to be tested was injected subcutaneously either in Freund's incomplete adjuvant or without adjuvant. After different time intervals (5 hr; 5 days) the skin from the tail of C_3H mice was grafted to $C_{57}Bl/6$ mice. The appearance of the total necrosis indicated the day of rejection.

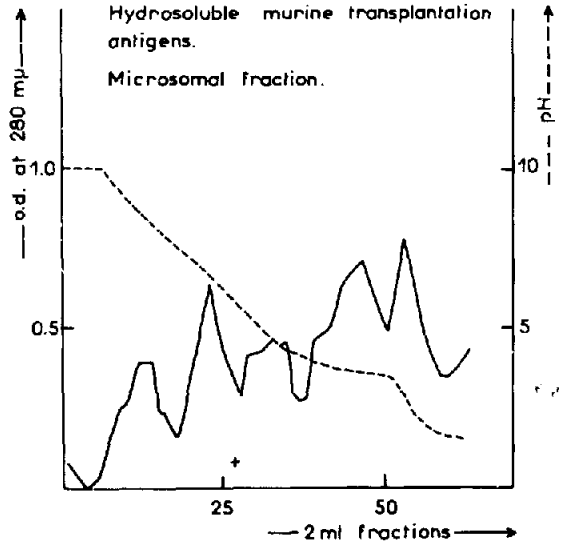

Fig. 1. Isoelectric focusing between pH 3 and 10 of the hydrosoluble fraction obtained by autodigestion from BP8 murine (C_3H) tumoral cells, microsomal fraction. —— absorbancy at 280 mμ; – – – pH; + biologically active fraction.

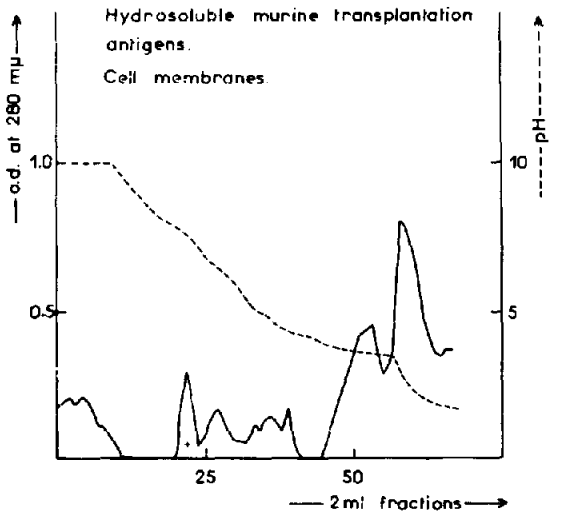

Fig. 2. Isoelectric focusing of the membrane fraction (for details, see fig. 1).

The hydrosoluble fraction was obtained by autodigestion (0.05 M tris-HCl buffer; pH 7.7; 3 hr; 37°; absence of enzymes) of the lipoprotein fraction.

2.1. *Isoelectric focusing in sucrose gradient column*

An LKB 8101 electrofocusing column of 110 ml capacity was used for these experiments. The carrier ampholyte (Ampholine LKB) was selected to give a pH gradient between pH 3 and pH 10. Preparation of the solutions and of the density gradient were performed as described in the preliminary instruction sheet and its addendum supplied by LKB Instruments [9]. The sample was prepared by adding 1 ml of the hydrosoluble fraction in the 12th and 13th fraction of the gradient; the whole column contains 24 fractions of 4.6 ml. Electrofocusing was performed for 48 hr with a potential of 600 V at 10°. The column was drained slowly through the bottom tubing. Fractions of 2 ml were collected and the absorbance of each fraction was determined at 280 mμ with a Beckman DU spectrophotometer.

Acrylamide gel electrophoresis was performed at pH 8.9 (0.025 M tris buffer), 25 min, 5.5 mA, 220 V.

3. Results

3.1. *Purification of a biologically active hydrosoluble fraction by isoelectric focusing*

5 Different assays with the hydrosoluble fraction obtained from the membranes and 2 different assays with the hydrosoluble fraction obtained from the microsomes of BP8 tumoral C_3H mice were achieved. The results were reproducible. Figs. 1 and 2 show the results of electrophoreses; the pH and the absorbance at 280 mμ of the eluates are reported.

3.2. *Biological activity*

The biological activity (*in vitro* and *in vivo*) of each fraction was tested after dialysis at 4° against 0.01 M tris buffer at pH 7.7. Only two or three 2 ml fractions were active *in vitro*; they were focused between pH 6.3 and 6.7. The specific activity of the material at the beginning was 150 μg/ml and that of the fraction after isoelectric focusing 65 μg/ml.

100 μg of the hydrosoluble active (*in vitro*) material obtained from the membranes after isoelectric focusing were injected in Freund's incomplete adjuvant 5 hr before the skin graft; the total necrosis appeared after 20.8 days; normal mice which were not

Fig. 3. Acrylamide gel electrophoresis of the active material obtained with the membrane fraction after isoelectric focusing (see fig. 1).

immunized rejected the skin graft after 15.8 days; thus a significant prolongation ($p < 0.01$) of the graft was observed.

100 μg of the same material obtained after isoelectric focusing were injected 5 days before the skin graft in the absence of adjuvant; the prolongation of the tolerance of the grafts was of 7.3 days (significant for $p < 0.001$).

100 μg of the initial hydrosoluble material which was not submitted to isoelectric focusing were injected in the same conditions; this material was not active *in vivo*.

3.3. *Acrylamide gel electrophoresis of the active fraction obtained by isoelectric focusing*

The active material obtained by isoelectric focusing from the membranes was submitted to acrylamide gel electrophoresis. Fig. 3 indicates the pattern obtained with the 2 ml fraction which has the highest activity; usually only one band could be observed. The identification of the H-2k specificities which may be present has not been undertaken. Very slight traces of two other bands seem to be present from time to time. The purified active material is quite stable and can be dialyzed, concentrated and endure low temperature ($-20°$) without loss of activity.

References

[1] B.D.Kahan, Proc. Natl. Acad. Sci. U.S. 53 (1965) 153.
[2] D.A.L.Davies, Immunology 11 (1966) 115.
[3] D.A.L.Davies, J.Colombani, D.C.Viza and J.Dausset, Biochem. Biophys. Res. Commun. 33 (1968) 88.
[4] B.D.Kahan, E.C.Holmes, R.A.Reisfeld and D.L.Morton, J. Immunol. 102 (1969) 28.
[5] D.A.L.Davies, B.J.Alkins, E.A. Boyse, L.J.Old and E.Stockert, Immunology 16 (1969) 669.
[6] O.Halle-Pannenko, J.-P.Lammelin, J.-L. Amiel, G.Mathé and P.Jollès, Compt. Rend. Acad. Sci. Paris 262 (1966) 531.
[7] O.Halle-Pannenko, N.Kiger, M.-C.Martyré and P.Jollès, Rev. franç. Etudes Clin. Biol. 14 (1969) 288.
[8] P.A.Gorer and Z.B.Mikulska, Cancer Res. 14 (1964) 651.
[9] LKB Instruments Inc., "LKB 8102 Electrofocusing column – Preliminary Instruction sheet and Addendum", Rockeville, Maryland (1967).