Characteristics of the cell surface antigen, p72, associated with a variety of human tumours and mitogen-stimulated T-lymphoblasts

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The monoclonal antibody 791T/36, prepared against a human osteogenic sarcoma cell line, 791T, reacts with a variety of human tumours and also mitogen-stimulated PBMN cells. The target antigen as expressed upon 791T cells is a monomeric plasma membrane-associated glycoprotein with an apparent M_r of 72000. By quantitative flow cytofluorimetry, approx. 10^5 antibody molecules bound per cell to T-lymphoblasts induced with PHA or Con A, whereas only a few thousand antibody molecules bound per cell to unstimulated cells, so that the antigen may be classified as a lymphocyte activation antigen. On lymphoblasts, the 791T/36 again reacted with a protein with an apparent M_r of 72000. This antigen therefore has a dual role as a tumour marker and lymphocyte activation antigen which may be implicated in the regulation of cell proliferation.

Human osteogenic sarcoma antigen *Lymphocyte activation antigen*

1. INTRODUCTION

The murine monoclonal antibody, 791T/36 (IgG2b), originally prepared against the human osteogenic sarcoma cell line, 791T, reacts with a number of human tumour cell lines [1] and after radioiodination, localizes within tumours in patients with a variety of malignancies, including colo-rectal tumours [2] and osteogenic sarcomas [3]. The antibody has also been employed as a vehicle for cytotoxic drugs for the development of new therapeutic agents showing selective cytotoxicity against tumour cells [4,5].

Biochemical analysis of the antigen defined by the 791T/36 antibody has been initiated with a view to elucidate the nature and characteristics of this cell surface molecule [6] and the recent demonstration that the antibody reacts with PHA-stimulated PBMN cells, but not unstimulated cells [7] offers additional opportunities for evaluating the functional significance of the expression of this antigen. This paper describes recent developments in parallel studies on the 791T/36 antibody defined antigen as expressed upon the osteogenic sarcoma cell line 791T and mitogen-stimulated T-lymphoblasts.

Abbreviations: PHA, phytohaemagglutinin; PBMN cells, peripheral blood mononuclear cells; FCS, foetal calf serum; Con A, concanavalin A; NP40, Nonidet-P40; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; PMSF, phenylmethylsulphonyl fluoride; WGA, wheat germ agglutinin

2. MATERIALS AND METHODS

2.1. *Cells*

Human osteogenic sarcoma 791T cells were grown as monolayer cultures in Eagle's minimum essential medium supplemented with 10% FCS. Cells were harvested following brief exposure to 0.25% trypsin.

PBMN cells were prepared from heparinized blood by discontinuous gradient centrifugation

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upon Ficoll-Triosil (Lymphoprep, Flow Laboratories, Irvine, Scotland). Stimulation with PHA (Wellcome Reagents, Beckenham, England) and Con A (Sigma, Poole) at final concentrations of 5 and 10 μg/ml, respectively, was performed employing culture conditions as in [7].

2.2. *Monoclonal antibody*

Hybridoma 791T/36 Clone 3 [1] provided the source of antibody from in vitro cultures. Antibody was purified by affinity chromatography using Sepharose–protein A (Pharmacia, Uppsala) [7].

2.3. *Radioimmunoprecipitation of 791T/36 defined antigens*

791T osteogenic sarcoma cells and PBMN cells cultured with and without PHA were surface labelled with ^{125}I ($Na^{125}I$, Amersham International, England) by lactoperoxidase-catalysed radioiodination [8] using approx. 1 mCi $^{125}I/10^7$ cells. Detergent (0.5% NP40, BDH) lysates were prepared and immunoprecipitated antigens were isolated following the sequential addition of antibody (10 μg) and Sepharose–protein A [6]. Analysis of antigens collected on antibody-Sepharose–protein A was performed by SDS–PAGE and autoradiography [6]. Enzymic treatment of radiolabelled immunoprecipitates with *Clostridium perfringens* neuraminidase, papain and chymotrypsin (each from Sigma) was performed as described in the text.

2.4. *Flow cytofluorimetry*

Cells treated with FITC-labelled 791T/36 antibody [6] added at saturation were analysed by flow cytometry using an FACS IV cell sorter (Becton Dickinson, Sunnyvale, CA). Excitation was at 120 mW at 488 nm from an argon ion laser and fluorescence was collected via a 10 nm band-pass filter centred at 515 nm. Appropriate forward angle scatter gating was used to confine analysis to viable cells or subpopulations of viable cells. Linear amplification was used to quantify mean fluorescence intensity and fluorescence signals were also amplified logarithmically for direct comparison of fluorescence intensity. The results were expressed as the channel number which corresponded to the mean fluorescence intensity per cell. Each unit increase in the channel number represents the signal acquired from 2228 molecules of fluorescein (to be published) so that with FITC-labelled antibody-treated cells the mean number of antibody molecules bound per cell may be calculated using the FITC:IgG molar ratio.

3. RESULTS AND DISCUSSION

The antigen precipitated by the monoclonal antibody 791T/36 from detergent lysates of ^{125}I-labelled 791T cells migrated in SDS–PAGE slab gels with an apparent M_r of 72000 (fig.1, lane 1). Both reduced and non-reduced samples gave equivalent results so that the antigen is a

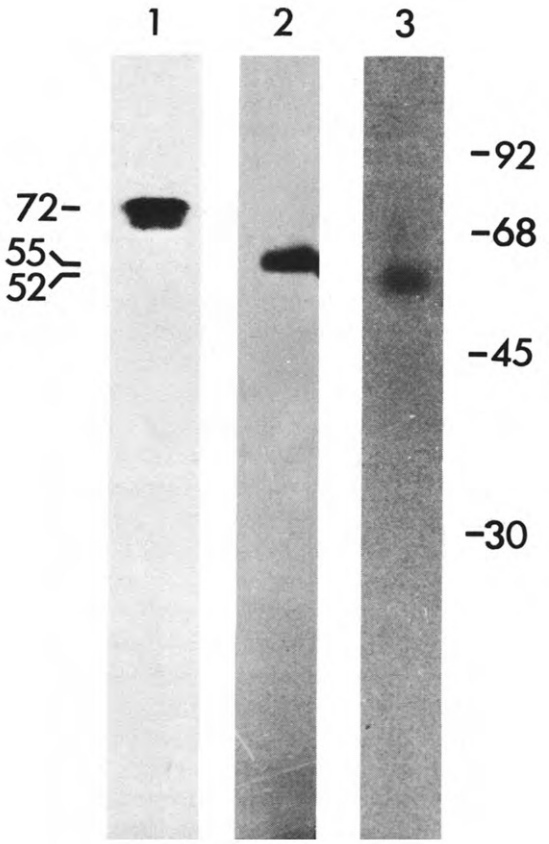


Fig.1. SDS–PAGE analysis of immune precipitates prepared by the addition of 791T/36 antibody and Sepharose–protein A to NP40 lysates of radiolabelled 791T tumour cells. Lane 1, untreated immune precipitate; lane 2, immune precipitate treated with *C. perfringens* neuraminidase; lane 3, immune precipitate treated with papain. The positions of M_r marker proteins are indicated to the right of the gel.

monomeric cell surface protein. As shown in fig.1, lane 1, the relatively broad band suggests that the antigen may be glycosylated since microheterogeneity in the saccharide residues would account for this observation. When immunoprecipitates were treated with neuraminidase (which had been pretreated with 1 mM PMSF) at up to 30 μg enzyme per immunoprecipitate, the apparent M_r was reduced to 55000 (fig.1, lane 2) indicative of extensive glycosylation, and since in repeated tests, microheterogeneity in the band of precipitated material was lost following neuraminidase treatment, this microheterogeneity was attributable to variations in sialic acid content.

That the p72 antigen is glycosylated was confirmed by metabolically labelling tumour cells with [^{3}H]glucosamine and again precipitating a 72-kDa antigen. Furthermore, using immunoadsorbent-purified p72 antigen, the molecule bound strongly to WGA, although less to Con A and not to lentil lectin (to be published).

Following treatment of immunoprecipitates with papain (5 μg/sample) a major 52-kDa polypeptide was generated (fig.1, lane 3). This also contained sialic acid since neuraminidase treatment reduced the apparent M_r to 50000. This fragment retained the epitope for the 791T/36 antibody. In comparable tests with chymotrypsin, it was again only possible to generate a large 47-kDa polypeptide which was resistant to further proteolysis. The findings suggest that only a limited portion of the 72-kDa glycoprotein antigen is susceptible to proteolysis the remainder being resistant to degradation in spite of a large excess of enzyme over substrate. This may be attributable to the molecule being heavily glycosylated as inferred from the results of neuraminidase treatment.

The 791T/36 monoclonal antibody also reacts with PBMN cells stimulated with the T-cell mitogen, PHA [7]. Fig.2 illustrates this by showing 3-dimensional displays generated by the FACS IV cell sorter in analyses of the reaction of FITC-labelled antibody with PBMN cultured for 3 days with and without PHA (lower and upper, respectively). With PHA-stimulated cultures, a large proportion of cells exhibited high forward angle light scatter and these corresponded to T-lymphoblasts. This was confirmed by morphological examination of cells sorted on the basis of their light scatter properties. Material with low light scatter near the

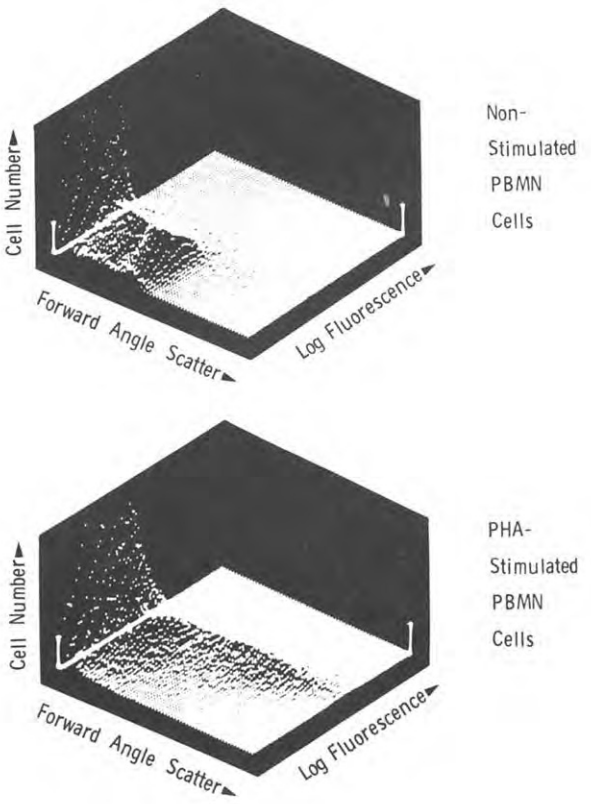


Fig.2. FACS IV cell sorter analyses. Three-dimensional displays of non-stimulated PBMN cells (upper) and PBMN cells cultured with PHA for 3 days (lower). Cells were treated with FITC-labelled 791T/36 antibody added at saturation (1 μg antibody protein/10^5 cells). The displays show the distribution of cells as a function of their surface fluorescence (i.e., uptake of FITC-791T/36 antibody) and their light scatter which is a measure of cell size.

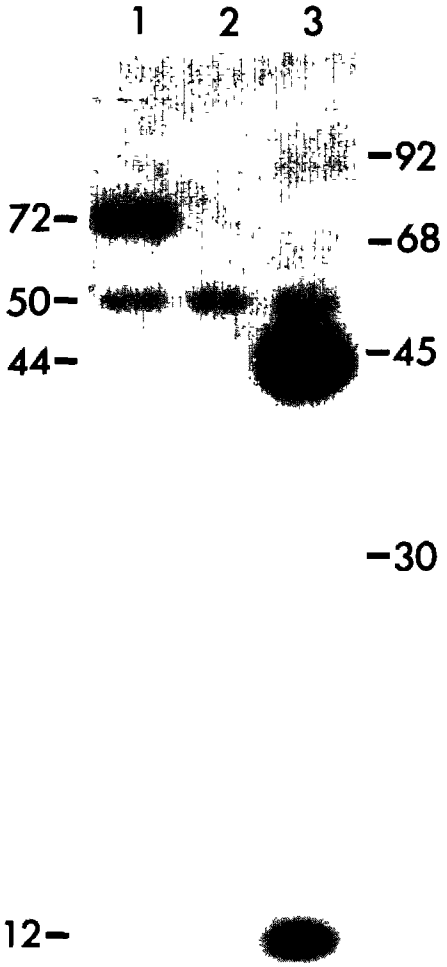
origin (fig.2) represents non-viable cells and cell debris. The T-lymphoblasts in PHA-stimulated PBMN cell cultures exhibited increased fluorescence and using the gating facilities on the FACS IV cell sorter, forward angle scatter gates were set around the T-lymphoblast subpopulation thus excluding small lymphocytes. The number of antibody molecules binding per lymphoblast was determined from the mean fluorescence of this subpopulation. As shown in table 1, T-lymphoblasts induced by PHA or Con A bound around 10^5 antibody molecules per cell compared with lymphocytes from non-cultured or cultured but non-stimulated PBMN cell preparations which

Table 1

Binding of FITC-labelled 791T/36 antibody to mitogen-stimulated PBMN cells as assessed by quantitative flow cytofluorimetry

Cell subpopulation analysed	Percentage of cells in subpopulation analysed (mean ± SD)	Number of FITC-791T/36 antibody molecules binding/cell ($\times 10^{-5}$) (mean ± SD)
Small lymphocytes from non-stimulated PBMN cell preparations	93.4 ± 3.8 (n = 18)	$\leqslant$0.1 (n = 18)
Lymphoblasts from PBMN cell preparations cultured with PHA for 3 days	45.5 ± 22.2 (n = 17)	1.1 ± 0.4 (n = 17)
Lymphoblasts from PBMN cell preparations cultured with Con A for 3 days	60.6 ± 12.0 (n = 6)	0.9 ± 0.5 (n = 6)

The number of determinations (n) is given in parentheses



bound, as far as could be accurately measured, only a few thousand antibody molecules per cell.

From these data, it may be concluded that the 791T/36 antibody defines a T-cell activation antigen upon mitogen-stimulated lymphoblasts. To compare this antigen with that expressed upon tumour cells (which bind approx. 10^6 antibody molecules per cell), radio-immunoprecipitation tests and SDS–PAGE analyses were performed using stimulated and non-stimulated PBMN cells. As shown in fig.3 the 791T/36 antibody precipitated a protein with an apparent M_r of 72000 which was equivalent to that precipitated from tumour cells.

While this p72 antigen is a useful marker for solid tumours in diagnostic studies [2,3] and for targeting drug-antibody conjugates [4,5], the find-

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Fig.3. SDS–PAGE analysis of immune precipitates prepared by the addition of antibodies and Sepharose–protein A to NP40 lysates of radiolabelled PBMN cells which had been cultured with PHA for 3 days. The antibodies used were 791T/36 monoclonal antibody (lane 1), normal mouse IgG (lane 2) and W6/32 monoclonal antibody (lane 3). The latter antibody, added as a positive control, defines HLA-A, B, C (shared determinant) and as shown, precipitates the HLA heavy chain (44 kDa) and β_2-microglobulin (12 kDa). The common band with an apparent M_r of 50000 corresponds to the heavy chain of cell surface IgG associated with B-cells in the PBMN cell, which is precipitated by Sepharose–protein A. The 791T/36 antibody failed to precipitate any protein with an apparent M_r of 72000 from freshly prepared PBMN cells or from PBMN cells cultured for 3 days in the absence of PHA.

ing that it also appears on T-lymphoblasts suggests that it may have a more important biological role. It is unlikely that the antigen is merely a marker of cellular proliferation per se since its expression has not yet been found to be elevated on PBMN cells from a small number of patients (22) with a variety of leukaemias and infectious mononucleosis (M.R. Price and T.E. Blecher, unpublished). These have included acute lymphoblastic leukaemias (T-cell, Null-cell, ALL-transformation in chronic myeloid leukaemia), prolymphocytic leukaemia, myeloblastic leukaemias (primary, relapsed, and AML-transformation in myelosclerosis) and acute myelomonoblastic leukaemia. Negative results have also been obtained with the circulating lymphocytes of chronic lymphocytic leukaemia, and the 'blood overspill' abnormal cells of cases of non-Hodgkin's lymphoma, myelomatosis and Waldenström's macroglobulinaemia. In addition, the abnormal mononuclear cells of 4 cases of Paul-Bunnell-positive infectious mononucleosis have also given negative results. Many of these cells are transformed or transforming T-lymphocytes.

Other lymphocyte activation antigens have been identified as receptors for essential protein nutrients required to support growth of a rapidly expanding cell population (e.g., transferrin receptors [9]) or as receptors for polypeptide hormones such as interleukin-2 required for maintaining the clonal expansion of T-cells primed with antigen or mitogen [10]. The present results emphasise the importance of defining the characteristics and functional role of the p72 antigen since the findings may have dual relevance to an understanding of growth in malignancy and to the regulation of lymphocyte proliferation in response to mitogen or antigen stimulation and thus be implicated in controlling the tempo or magnitude of T-cell immune responses.

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