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Reduction of serum X-prolyl dipeptidyl-aminopeptidase activity in tumour-bearing mice and reversal of reduced enzyme activity by lentinan, an anti-tumour polysaccharide

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Summary. Serum X-prolyl dipeptidyl-aminopeptidase activity which had been shown to be depressed in cancer patients was clearly reduced in mice with Ehrlich carcinoma and Sarcoma 180, and slightly reduced in mice with methylcholanthrene-induced sarcomas. The reduced enzyme activity was completely reversed during tumour regression of sarcoma 180 by administration of lentinan, which causes regression of sarcoma 180.

X-Pro dipeptidyl-aminopeptidase (dipeptidyl-aminopeptidase IV), which hydrolyzes N-terminal X-Pro from peptides, is a dipeptidyl-aminopeptidase discovered in the rat liver and kidney by Hopsu-Havu and Glenner[1] by using a chromogenic substrate, Gly-Pro β-naphthylamide. This enzyme was found in the serum of various animals including the human[2-4]. The enzyme activity in the human serum was found to be decreased in patients with gastric cancer, pancreatic cancer, acute lymphocytic leukemia, lymphosarcoma and Hodgkin's disease, with Gly-Pro β-naphthylamide or X-Pro p-nitroanilides (X= Gly, Ala, Lys, Arg, Glu, and Asp) as substrate[3,5,6]. In order to clarify the significance of a decrease in the serum enzyme activity of cancer patients, we have examined the changes in serum X-Pro dipeptidyl-aminopeptidase activity in tumour-bearing mice with and without the administration of lentinan, a potent anti-tumour polysaccharide[7].

Materials and methods. C3H/CRJ and ICR/CRJ mice (females) were used for experiments. Lentinan, a β-(1 → 3) glucan isolated from a mushroom *Lentinus edodes* (Berk.) Sing., was prepared at Central Research Laboratories, Ajinomoto Co., INC. Autochthonous tumour was induced by s.c. administration of methylcholanthrene to C3H/CRJ mice at 11 weeks of age, and the serum enzyme activity was assayed at 28 days after the 1st observation of the tumours, the size of which being about 0.5 cm in diameter or 20 mm^2 in volume. Transplantable tumour-bearing mice were prepared with ICR/CRJ mice by transplantation of Ehrlich ascites carcinoma or Sarcoma 180 tumour (3×10^6 cells per mouse). Ehrlich ascites carcinoma was transplanted s.c. at 5 weeks of age, and mice were killed at 35 days after transplantation. Sarcoma 180 tumour cells were inoculated s.c. to 2 groups of mice at 5 weeks of age, then mice of 1 group were injected i.p. with 25 µg/mouse of lentinan daily for 10 days starting from day 1 after tumour inoculation, while the mice of the other group were not treated with lentinan. Mice were killed at 14, 21, 28 and 35 days after transplantation. We initially planned the experiments to consist of at least 4 mice for each group. However, we reduced the numbers of mice to 3 in Sarcoma 180 tumour-bearing mice in order to see the time course of the effects of lentinan. The diameter of the tumours was measured by vernier calipers and the surface area was calculated. X-Pro dipeptidyl-aminopeptidase activity was assayed by using a highly sensitive fluorescence assay with a newly synthesized substrate, 7-(Gly-Pro)-4-methylcoumarinamide[8,9]. The incubation mixture for X-Pro dipeptidyl-aminopeptidase contained (total volume 100 µl) 60 mM glycine-NaOH buffer (pH 8.7), 0.5 mM 7-(Gly-Pro)-4-methylcoumarinamide tosylate, and 10 µl of serum containing the enzyme. Serum was omitted during the incubation and added after stopping the incubation for the control tube. Incubation was carried out at 37 °C for 30 min, the reaction was stopped by adding 3.0 ml of 1 M sodium acetate buffer, pH 4.2, and the fluorescence intensity of 7-amino-4-methylcoumarin formed was read at 460 nm with excitation at 380 nm. In order to see whether or not the changes in serum X-Pro dipeptidyl-aminopeptidase activity are specific, the activity of serum leucine aminopeptidase, a similar enzyme with a broader substrate specificity, was also examined. Leucine aminopeptidase activity was assayed by using leucine β-naphthylamide as substrate, as described previously[10].

Results and discussion. The activities of X-Pro dipeptidyl-aminopeptidase and leucine aminopeptidase in the sera of tumour-bearing mice are shown in the table. The values of X-Pro dipeptidyl-aminopeptidase activity of normal mouse in the table are much higher than those described previously for the mouse (Nagatsu et al.[2]). The reason of this difference may be due to the differences in the mice strains and/or in the assay conditions. This assay was done at pH 8.7 (an optimum pH) with 7-(Gly-Pro)-4-methylcoumarinamide as substrate, whereas in the previous assay[2] Gly-Pro β-naphthylamide at pH 7.0 was used. Different normal values were also observed in the 2 mice strains under the same assay conditions in this study, indicating a strain difference of the enzyme activity.

A small reduction in serum X-Pro dipeptidyl-aminopeptidase

Activities of X-Pro dipeptidyl-aminopeptidase and leucine aminopeptidase in the sera of tumour-bearing mice and the effect of lentinan, an anti-tumour polysaccharide

Mice	Age (weeks)	Experiment	Number of mice	Size of tumours (mm^2) mean ± SEM	X-Pro dipeptidyl-aminopeptidase activity (nmoles/min/ml serum ± SEM)	(% control)	Leucine aminopeptidase activity (nmoles/min/ml serum ± SEM)	(% control)
C3H/CRJ (♀)	15	Normal (control)	5	0	18.59 ± 0.25	100	15.3 ± 0.53	100
	15	Methylcholanthrene-induced cancer-bearing (28 days)	5	314 ± 144	14.63 ± 1.89	79	17.1 ± 0.53	112
ICR/CRJ (♀)	10	Normal (control)	10	0	28.00 ± 0.90	100	18.1 ± 0.6	100
	10	Ehrlich tumour-bearing (35 days)	4	855 ± 236	10.98 ± 0.52	39	14.9 ± 0.8	82
	7	Sarcoma 180 tumour-bearing (14 days)	3	195 ± 58	20.46 ± 1.17	73	17.4 ± 0.8	96
	7	Sarcoma 180 tumour-bearing (14 days) and lentinan-treated	3	86 ± 35	14.88 ± 3.12	53	16.9 ± 1.5	93
	8	Sarcoma 180 tumour-bearing (21 days)	3	372 ± 134	12.21 ± 0.32	44	15.4 ± 0.9	85
	8	Sarcoma 180 tumour-bearing (21 days) and lentinan-treated	3	0	20.22 ± 3.82	72	19.1 ± 1.4	105
	9	Sarcoma 180 tumour-bearing (28 days)	3	597 ± 282	13.32 ± 1.53	48	14.0 ± 0.3	77
	9	Sarcoma 180 tumour-bearing (28 days) and lentinan-treated	3	0	28.20 ± 1.80	101	21.8 ± 0.8	120
	10	Sarcoma 180 tumour-bearing (35 days)	5	954 ± 316	12.06 ± 1.04	43	16.2 ± 1.7	90

In parentheses: the day after tumour induction by methylcholanthrene or after tumour transplantation in the donor mice.

activity was observed in mice with methylcholanthrene-induced cancer. In contrast, serum leucine aminopeptidase activity was not decreased, but slightly increased.

The serum X-Pro dipeptidyl-aminopeptidase activity was more significantly decreased in mice with the transplantable tumours (Ehrlich carcinoma and Sarcoma 180) than in mice with methylcholanthrene-induced autochthonous tumours.

A substantial decrease in serum X-Pro dipeptidyl-aminopeptidase activity was clearly observed in Ehrlich carcinoma-bearing mice (39% of the control mice), whereas a reduction in serum leucine aminopeptidase activity was much smaller than in serum X-Pro dipeptidyl-aminopeptidase activity.

A pronounced decrease in serum X-Pro dipeptidyl-aminopeptidase activity was also observed in Sarcoma 180 tumour-bearing mice, and the time course of the changes in the serum activities of X-Pro dipeptidyl-aminopeptidase and leucine aminopeptidase during the tumour development was followed. As shown in the table, serum X-Pro dipeptidyl-aminopeptidase activity in Sarcoma 180 tumour-bearing mice decreased progressively down to about 40% of the level in control mice. The decrease in the serum enzyme activity was clear-cut and rapid in the initial 21 days of tumour development. Serum leucine aminopeptidase activity also had a tendency of a slight decrease especially at 21–28 days of tumour growth, but at 35 days after transplantation the decrease was not clear-cut. In contrast, serum X-Pro dipeptidyl-aminopeptidase activity of the tumour-bearing mice was greatly reduced down to 43% of the level of control mice at 35 days after transplantation. In lentinan-treated mice, the reduction in serum X-Pro dipeptidyl-aminopeptidase activity was more pronounced at 14 days after transplantation, but the enzyme activity was reversed from 21 days when the tumour began to disappear, and at 28 days after transplantation when the tumours disappeared, the enzyme activity was reversed completely.

The possibility of formation of some endogenous inhibitors of X-Pro dipeptidyl-aminopeptidase in the sera of the tumour-bearing mice was examined by adding homogeneous enzyme purified from the human submaxillary gland[1] to the sera, and nearly 100% of the activity could be accounted for. Mixing the sera from tumour-bearing mice with those from controls also gave additive activity. This result indicates that the decrease in the serum enzyme activity was not due to the formation of endogeneous inhibitors in the sera of the tumour-bearing mice.

The results suggest a specific decrease of serum X-Pro dipeptidyl-aminopeptidase activity in tumour-bearing mice. Complete reversal of reduced serum enzyme activity by the administration of lentinan in tumour-bearing mice could be due to the removal of unknown effects of tumour on the level of the serum enzyme during the process of tumour regression. The difference between mice with methylcholanthrene-induced tumours and controls was much smaller than in mice with the transplantable tumours. This may be due to the differences between strains of animals and/or tumours. More differentiation concerning differences between strains of animals and tumours should be examined in future studies.

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Association between pregnancy-associated α_2-glycoprotein (α_2-PAG) and mixed leucocyte reaction determinants on the leucocyte surface

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Summary. α_2-PAG is present on the surface of mononuclear blood leucocytes and can be demonstrated predominantly on B-lymphocytes and monocytes. Pretreatment of cells with antibody to α_2-PAG leads to a marked reduction in Fc-rosette formation. Competitive blocking experiments with specific antisera reveal a particularly close association between α_2-PAG and MLR (mixed leucocyte reaction) determinants on the cell surface. These findings suggest one mechanism whereby α_2-PAG may modify cell-mediated immune responses.

Modification of cell-mediated immunity by factors present in serum is thought to play some part in tolerance of the pregnant female towards her foetus[1-4]. Prominent among the substances to which such an effect has been attributed is pregnancy-associated α_2-glycoprotein (α_2-PAG) a macroglobulin of mol. wt about 300,000[5], present, in trace amounts, in all normal sera[6,7] and which appears to be a leucocyte product[8]. The serum level of this protein rises dramatically during pregnancy[5,9] and raised levels have also been reported in patients with disseminated malignancies[10-12]. It has apparent immunosuppressive properties in vitro, i.e. reduces antigen and phytomitogen-induced lymphocyte transformation[13,14], blocks the mixed leucocyte reaction (MLR)[15] and impairs macrophage electrophoretic mobility[16].

To understand the mechanism whereby α_2-PAG exerts its influence on cellular immune reactions, it is essential to determine the distribution of the protein on human leucocytes and its relationship to cell surface determinants already characterized. Recent studies have shown that α_2-PAG can be identified on human B-lymphocytes and monocytes[17,18]. These cells are also known to carry the surface determinants (HLA-D) responsible for stimulation in mixed leucocyte cultures[19,20]. There is a close spatial relationship between serologically determined HLA-D related antigens (the 'DRW' series, formerly 'Ia') and the Fc receptor site on B-lymphocytes[21]. An association between α_2-PAG and the products of the HLA-DR locus is therefore suggested by the demonstration that pretreatment of mononuclear cells with anti-α_2-PAG antibody markedly reduces the number of Fc-rosette-forming cells[17].

In the present study we have extended these observations and have compared the distribution of α_2-PAG and other surface determinants on human leucocytes.

Materials and methods. Mononuclear cells were obtained from 10-ml samples of EDTA-treated peripheral blood by centrifugation on Ficoll-Triosil[22]. 'E'-rosettes were formed by incubating the cells overnight at 4 °C with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes[23]. Rosetting and nonrosetting cells were then separated by repeating the centrifugation on Ficoll-Triosil. Red cells were removed by osmotic lysis.

Direct and indirect immunofluorescence staining for α_2-PAG were carried out as previously described[17,18] using a commercial rabbit anti-α_2-PAG IgG (Dakopatts) and goat antirabbit gamma globulin (Nordic). Fc-receptor-bearing cells were demonstrated by rosette-formation with IgG-coated ox erythrocytes[24,25]. The inhibitory effect of anti-α_2-PAG on Fc-rosette formation was demonstrated using either intact IgG or F (ab')$_2$ fragments of the antibody[26].

The following commercially-available rabbit antisera to human cell surface determinants were used: from Dakopatts, anti-IgM (μ-chain specific) and anti-β_2 microglobulin (β_2M); from Sera-Lab, anti-HLA (all specificities) and anti-Ia ('DRW'). In addition, rabbit antisera were raised against partially-purified surface membranes of human B lymphoblastoid cell lines. One of the lines used was EB_1, which carries HLA, β_2M and 'DRW' determinants but which does not stimulate in mixed leucocyte culture and hence appears to lack the MLR antigen product of the HLA-D locus[27]. Anti-EB_1 serum was extensively absorbed with T-cell lines ($MOLT_4$ and CCRF-CEM) before use. In microcytotoxicity and indirect immunofluorescence tests this antiserum behaves like an anti-DRW reagent i.e. it is highly reactive with all B-cell lines and with peripheral blood B-cells but not with T-cells. Rabbit antiserum was also raised to membranes from a second B-cell line, DAUDI. This line does not produce β_2M[28] and hence does not carry surface HLA, A-, B- or C-determinants. It does express DRW antigens and is a potent stimulator in mixed leucocyte cultures[27]. Anti-DAUDI serum was extensively absorbed with T-cell lines and with EB_1 cells in an attempt to derive a reagent which is specific for MLR determinants. In microcytotoxicity assay the resultant 'anti-MLR' serum is relatively weakly reactive with peripheral blood B-cells and with all B-cell lines (except EB_1, with which it is unreactive[29]). A similar level of activity is observed on indirect immunofluorescence testing; yet at greater dilutions the antiserum retains MLR-blocking activity while, unlike anti-DRW reagents, it does not inhibit mitogen-induced lymphocyte activation[29]. The Student t-test was used for statistical analysis of results.

Results and discussion. Table 1 shows that α_2-PAG can be identified on the surface membranes of mononuclear cells and that it is predominantly associated with those cells which do not form 'E'-rosettes (non-T-cells). Pretreatment with unlabelled specific antibody to α_2-PAG resulted in a 20% reduction ($p < 0.025$), with respect to the control value,