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EXPRESSION OF HANGANUTZIU-DEICHER ANTIGEN IN ACTIVATED HUMAN T LYMPHOCYTES

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SUMMARY: Hanganutziu-Deicher (HD) antigen is a heterophile antigen that is widely distributed in many animals other than humans and chickens and is highly immunogenic in humans and chickens. In the present study, we demonstrated expression of HD-antigenic glycoproteins in activated T lymphocytes by SDS-PAGE and immunoblotting. Treatment with IL-2 plus PMA induced 29kD glycoprotein antigen detected under reducing condition. It contained sialic acid epitope of HD antigen because of the expression being neuraminidase-sensitive. Treatment with PMA plus A 23187 or PHA treatment and then PHA plus IL-2 treatment also induced two proteins of Mr 50kD and 70kD. These expressions were not detected in all individuals examined. These results indicate that HD antigen is an activated T cell antigen and expressed as an isoantigen as it is expressed in cancerous tissues from some patients.

Hanganutziu-Deicher (HD) antigen is a heterophile antigen in humans and its antigenic determinant is N-glycolylneuraminic acid (NeuGc), which is found on both glycolipids and glycoproteins. HD antigen is recognized by a heterophile antibody, HD antibody which is produced in sera of patients after therapeutic injection of foreign serum (1,2). This HD antigen was detected in malignant tissues from patients with various tumors (3), and recently, we demonstrated tumor associated HD antigen of glycosphingolipids in colon cancer (4) and melanoma (5) and retinoblastoma cell lines (6) and glycoproteins in a gastric cancer cell line (7) by thin-layer chromatography immunostaning or Western blotting and immunostaining . In this paper, we report the detec-

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ABBREVIATIONS: HD, Hanganutziu-Deicher; NeuGc, N-glycolylneuraminic acid; GSL, glycosphingolipid; HD3, II^{3}NeuGc-LacCer.

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tion of HD glycoprotein in activated T lymphocytes from some human volunteers.

MATERIALS AND METHODS

Isolation of Lymphocytes and Purification of T lymphocytes. Human blood in acid-citrated dextrose was obtained from the Japan Red Cross Osaka blood bank. Peripheral blood mononuclear cells (PBMC) were isolated from acid-citrated blood of a number of donors by Ficoll-Hypaque density gradient centrifugation . The T lymphocyte-rich fraction was purified by passage over a nylon wool column, after stimulation of the PBMC with T-cell activators.

Lymphocyte Culture for T cell stimulation. PBMC(2×10^6 cells/ ml) were cultured in serum-free medium, Celgrosser H (Meguro Res. Lab., Japan) or 2 % human serum from blood group AB donor (Flow Laboratories, Irvine, Scotland) in RPMI 1640 (Flow Lab.) with various T cell activators for several days under 37° C and 5% CO_2-containing humidified air. For T-cell activation, 4ug/ml of PHA (Difco laboratories, Mich) , 5ug/ml of PWM (E.Y. Laboratories, CA), 40 ng/ml of PMA (Sigma Chemical Co.), 100IU/ml of IL-2 (Genentech, Inc., CA) , and 250ng/ml of Calcium ionophore A 23187 (Sigma Chemical Co.), each or mixture of two reagents were used as indicated in figures and table.

Western blotting and Immunostaining. Samples of 1×10^7 PBMC and T lymphocytes were solubulized in 100-200 ul of 1% SDS-containing sample buffer, and the supernants after centrifugation at 100,000g for 30 min were subjected to electrophoresis in 4-20 % gradient polyacrylamide gel(SDS-PAGE) (8), Western blotting (9) and immunostaining with affinity-purified II^3NeuGc-LacCer (HD3) antibody from chicken antiserum, avidin-biotin-complex (ABC) and biotinylated alkaline phosphatase (10). Briefly, blotts on nitrocellulose membranes (Schleicher & Schuell, FDR) were immunostained by incubation with HD3 antibody at a concentration of 6 ug/ml in Tris basal saline (TBS: 0.1M Tris-HCl, 0.1N NaCl and 2mM $MgCl_2$, pH 7.5) at room temparature for 1h and then, with anti-chicken IgG conjugated with biotin (Vector laboratories, CA) in TBS at room temperature for 1h, and further incubated with streptavidin-conjugated alkaline phosphatase (Bethesda Res. Lab., MD) diluted 4000-fold with TBS (pH 9.5) containing 50 mM $MgCl_2$ at room temperature for 15 min. Finally, the enzyme was detected with a substrate solution of 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and nitroblue tetrazolium chloride (BCIP-NBT kit, BRL) diluted 200-fold with the same buffer. As a

negative control, normal chicken serum was used at 1500-fold dilution instead of specific antibody.

Neuraminidase treatment. Samples of 10ul of lymphocyte protein (1mg/ml in PBS) were mixed with 10ul of 0.01units of Streptcoccal neuraminidase (Seikagaku Kogyo Co., Japan) in PBS, incubated at 37°C for 2h and boiled for 5 min with an equal volume of 2-fold concentrated sample buffer before SDS-PAGE.

RESULTS

First PBMC were stimulated with each or mixture of IL-2 and PMA , and HD-antigenic protein expression was analyzed by SDS-PAGE followed by immunoblotting(Fig. 1). Treatment with combination of PMA and IL-2 gave expression of an HD antigen-active protein band of Mr 29kD in the reduced condition, although other non-specific protein bands which were reactive with normal chicken IgG were also detected (lane 5). The T cell-rich fraction

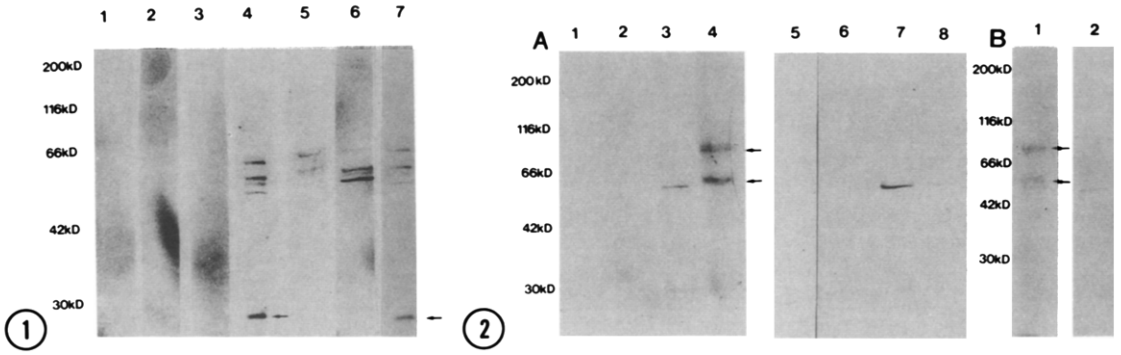

Fig. 1. HD antibody-reactive glycoproteins detected in PBMC stimulated with PMA and/or IL-2 by immunoblotting procedure. PBMC were treated without any stimulation (1) or with IL-2(2), PMA(3) or PMA plus IL-2 (4,5,6,7). PBMC(1,2,3,4,5,6) and T lymphocyte fraction(7) were solubilized with SDS and analyzed by SDS-PAGE using 4-20 % gradient polyacrylamide gel before (4) or after neuraminidase treatment (6), and the blotted paper was stained with chicken HD3 antibody (1,2,3,4,6,7) or normal chicken IgG (5) and ABC reagent. The arrows show the bands of HD antigen.

Fig. 2. Demonstration of the expression of HD-antigenic glycoproteins in PBMC stimulated with PMA plus A 23187 or PHA plus IL-2 by immunoblotting. PBMC were not stimulated (A-1,5) or stimulated for 12 days with IL-2 (A-2,6), PHA(A-3,7), PHA for 7 days and then PHA plus IL-2 for 5 days (A-4,8) or PMA plus A 23187 (B-1,2). The blots were probed with HD3 antibody (A-1,2,3,4 and B-1) or normal chicken IgG (A-5,6,7,8 and B-2). The arrows show the bands of HD antigen.

might be the main source of HD antigen, because it also showed the same antigenic band (lane 7) and T cell-deleted fraction showed no band(data not shown). Neuraminidase treatment removed the 29kD band, confirming that the antigenic epitope of the glycoprotein is sialic acid same as HD antigen determinant. This glycoprotein of 29kD was found only in culture of PBMC stimulated with PMA plus IL-2 for 7-10 days and bound to a WGA-agarose column (Seikagaku Kogyo, Japan)(data not shown). Other HD antigen-active glycoproteins were also demonstrated in PBMC stimulated with PHA alone for 7 days and then PHA plus IL-2 for 5 days, or PMA plus A 23187 for 12 days (Fig.2). The protein bands (50 kD and 70 kD)were similar in both cultures, while they appeared after 12 day culture in the PHA-IL-2 treatment, and 4 day culture in the PMA-A 23187 treatment. The former treatment was later used as a model for T cell activation studies. When both compounds were added together, no HD proteins were detected by the immunoblotting method. But after incubation with PHA alone for 7 days, addition of IL-2 affected on expression of HD antigen 5 days later. But the expression of the antigen was not detected in all individuals examined. The antigenic expression by PMA plus IL-2 treatment or PMA plus A 23187 as well as PHA and then PHA plus IL-2 treatment was detected in some of examination volunteers, the percentages of positive cells were 25 %, 29 %, and 40 %, respectively at an average in these positive cases(Table 1).

Table 1. Induction of HD-antigenic glycoproteins in PBMC by various treatments

Treatment	Positive No. / total No.[a]
None	0/15
PMA	0/5
PHA	0/10
PWM	0/5
PMA + PHA	0/5
PMA + IL-2	2/8
PMA + PWM	0/3
PMA + A 23187	1/5
PWM + IL-2	0/3
PHA + IL-2	0/3
PHA +(PHA+IL-2)[b]	2/5

a) HD antibody-reactive glycoprotein detected in treated PBMC by SDS-PAGE and Western Blotting.
b) The PBMC was firstly treated with PHA for 7 days and then with both PHA and IL-2 for 5 days.

DISCUSSION

HD antigens have been found in many animals other than humans and chickens. In humans and chickens, the synthesis of HD antigen epitope seems to be stopped or suppressed. HD antigen has also been demonstrated in avian Marek's disease lymphoma and human cancerous tissues with high frequency, suggesting that the negative control of the synthesis is occasionally broken during carcinogenesis. The present results indicate that synthesis of NeuGc occurs in activated T lymphocytes of some individuals. NeuGc synthesis from NeuAc is catalyzed by sialic acid monooxygenase and cytochrome b_5 (11) and HD antigenic glycoprotein is synthesized by sialyltransferase which recognizes core protein carbohydrate sequence and transfers NeuGc from CMP-NeuGc. But, there is no data so far on the core protein synthesis, the sialic acid monooxygenase or the sialyltransferase.

The HD antigen expression occurs in activated T cells as well as cancerous tissues from some individuals (not all individuals) like an isoantigen expression (12), although an isoantigen is expressed in normal tissues. It may be because the negative control of the antigen synthesis is broken at random in a genetical base or linking to some histocompatible expression.

Finally, the biological significance of HD antigen expression in activated T lymphocytes should be considered. The antigen may be secreted from activated T lymphocytes into blood stream and give the antibody response . In our preliminary studies, HD antibody was detected in sera from patients with autoimmune diseases or Lepra infection with high frequencies (unpublished data) . The finding suggests that the production of HD antigen in chronically activated T lymphocytes in autoimmune diseases or chronic inflammation diseases triggers antibody production.

In this paper, we showed that HD antigen is an activated T cell antigen and expressed like an isoantigen. Now, we are investigating the biological means of this antigen production in cell development and differentiation.

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