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ELEVATION OF HETEROPHILIC ANTIBODIES TO RABBIT ERYTHROCYTES IN HUMAN PATHOLOGIC SERA: QUANTITATIVE STUDIES BY ELISA USING A GLYCOSPHINGOLIPID ANTIGEN*[1]

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Human heterophilic hemagglutinin to rabbit erythrocytes was quantitatively determined by the hemagglutination test and enzyme linked immunosorbent assay (ELISA) using rabbit erythrocyte pentaglycosyl ceramide (CPH), Gal (α 1–3) Gal (β 1–4) GlcNAc (β 1–3) Gal (β 1–4) Glc-Cer as the antigen. The antibody levels in sera from 74 Hanganutziu-Deicher (H-D) antibody-positive patients were significantly higher than those in sera from 55 healthy donors, and the correlation between the antibody levels detected by ELISA and hemagglutinin titers was significant. The antibody levels detected by ELISA correlated to the IgG antibody levels, while the hemagglutinin titers correlated to the IgM antibody levels. These results suggested that IgG levels as well as IgM levels against rabbit erythrocytes were elevated in H-D antibody-positive patients with various tumors and infectious diseases. In 4 out of 67 cancer patients collected randomly, abnormally high levels of anti-CPH IgG antibody were detected.

Key Words: heterophile antigen, heterophile antibody, natural antibody, glycosphingolipid, rabbit erythrocyte

INTRODUCTION

A heterophilic hemagglutinin to rabbit erythrocytes was detected in healthy human

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**To whom correspondence should be addressed. Abbreviations used: NeuGc, N-glycolyneuraminic acid; Cer, ceramide (N-acylsphingosine); CPH (Pentaglycosyl ceramide), Gal (α 1–3) Gal (β 1–3) Gal (β 1–4) Glc-Cer; hematoside (NeuGc), NeuGc (α 2–3) Gal (β 1–4) Glc-Cer.

sera as well as in patient sera.[12] The hemagglutinin is different from anti-B isoantibody and other heterophilic antibodies such as Hanganutziu-Deicher (H-D) antibody[4] or Paul-Bunnell antibody.[11] Our previous paper[14] described the purification of an antigenic glycosphingolipid from rabbit erythrocyte stroma and identified the antigen as pentaglycosyl ceramide (CPH). In the present study, we attempted to quantify the serum antibody levels in patients and healthy persons by enzyme-linked immunosorbent assay (ELISA) using this compound and the hemagglutination test. It was found that the antibodies were significantly elevated in patients with cancer and other diseases.

MATERIALS AND METHODS

Antigenic glycosphingolipids and sera: The antigenic CPH of a heterophilic hemagglutinin to rabbit erythrocytes was previously purified,[14] and the H-D antigenic hematoside (NeuGc) was also prepared as described in another previous paper,[8] H-D antibody-positive sera from 74 patients with tumors (62%), infectious diseases (21%) and other diseases (17%) were screened from 1333 patients at Hokkaido University Hospital, Sapporo, by the hemagglutination test using equine erythrocytes (HA titer= 128). The details published in our previous communication.[7] Sera from 67 patients with various cancers, including 24 cases of gastric cancer, 13 of colorectal cancer, 7 of breast cancer, 6 of hepatoma, 5 of pancreatic cancer, 3 of lung cancer, 3 of gall bladder tumor and 6 of other tumors were obtained from Teikyo University Hospital, Tokyo. Normal human sera were obtained from apparently healthy adult individuals, who offered blood for blood transfusions after being diagnosed as normal by several biochemical examinations conducted at the Hokkaido Red Cross Blood Center. All sera were stored at –80°C before use.

Hemagglutination test: The test was performed as follows: a two-fold serial dilution of human serum (0.1ml) was mixed with 0.1ml of 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl (PBS) and the same volume of 1.4% rabbit erythrocyte suspension. After 1 h incubation at room temperature, each tube was centrifuged at 2000rpm for 2min and the hemagglutination was read after gentle shaking. The final dilution that caused hemagglutination was designated as the hemagglutination titer.

ELISA test: The test was performed following the method described in our previous paper,[14] with minor modifications. Prior to the test, each well of an EIA microtitration plate (Coster) was coated with 2.5 μg of the glycosphiagolipid antigen which was dissolved in 50 μl of methanol by allowing the methanol to evaporate. After blocking non-specific binding sites of the wells by incubation with 0.2ml of 1% egg albumin in PBS, 10–fold diluted test sera for CPH antigen, or 41–fold diluted sera for hematoside (NeuGc) antigen, were added to each well and allowed to react at 37°C for 2 h. After washing with 0.05% Tween 20 in PBS, the amount of bound antibody was determined by adding 50 μl of alkaline phosphatase-conjugated rabbit IgG antibody

to Fab fragment of human IgG[6], or 50 μl of 2000–fold diluted peroxidase-conjugated Fab fragment of goat IgG antibody to γ chain of human IgG (Medical Biological Lab., Japan). The reaction was allowed to stand at 37°C for 2 h, and then 200 μl of the substrate solution, 2.5mM p-nitrophenyl phosphate sodium salt and 1mM $MgCl_2$ in 0.05M sodium bicarbonate buffer (pH 9.7) for alkaline phosphatase, or 0.2mM 2,2'–azino-di– (3–ethylbenzothiazoline–6–sulfonic acid) diammonium salt and 0.004% hydrogen peroxide in 0.05M citrate buffer (pH4.0) for peroxidase were added. The enzyme reaction was allowed to stand at 37°C for 1h alkaline phosphatase, or at room temperature for 1h for peroxidase, and the amount of product was read by measuring the absorbance at 405nm using a Titertek Multiskan (Flow Lab., Inc.). This value was corrected after subtracting the amounts read in the wells without antigen from those with antigen. The ELISA value for each serum was expressed as a relative ELISA value (percentage) to that of a typical antibody-containing serum.

For more exact quantitative analyses of IgG and IgM antibodies to CPH, ELISA using 2–fold serial dilutions of test sera started from 5–fold dilution was performed for several sera. For separate titration of IgG and IgM antibodies, peroxidase-conjugated specific anti-μ chain of human immunoglobulin (Medical Biological Lab.), respectively, were used in the same amounts as described above. The area under the titration curve for the test serum was integrated and expressed as a percentage of that of a constant serum (E. S.) according to Sedgwick et al.[13]

Results

Heterophilic antibody levels to rabbit erythrocytes in normal sera from 55 healthy individuals and in pathologic sera from 74 H-D antibody-positive patients were quantitatively detected by ELISA using CPH as the rabbit erythrocyte antigen, as reported in our previous paper,[14] and the hemagglutination test. As shown in figure 1, the antibody levels were significantly higher in patient sera than in normal sera ($P < 0.001$), as detected by both ELISA and the hemagglutination test.

Correlation between the antibody levels detected by ELISA and hemagglutinin titers was significant only in patient sera ($p < 0.05$) and not in normal sera (Fig. 2).

As shown in figure 2, several sera had high ELISA values and low hemagglutinin titers, or vice versa. Thus in these sera, anti-CPH IgG and IgM antibody levels were determined, respectively, by ELISA using anti-IgG (γ) and anti-IgM (μ) as the 2nd antibody. The sera with high ELISA values contained higher anti-CPH IgG antibody levels, and that with high hemagglutinin titers contained higher anti-CPH IgM antibody levels (Tab. 1). Moreover, the distribution patterns of antibody levels in both groups of normal and patient sera detected by ELISA using anti-IgG (Fab) or anti-IgG (γ) as 2nd antibody were quite similar (data not shown). These results suggest that the ELISA values and hemagglutinin titers in figure 1 depend on the amount of anti-CPH IgG and IgM antibody, respectively, and that anti-CPH IgG as well as IgM antibody

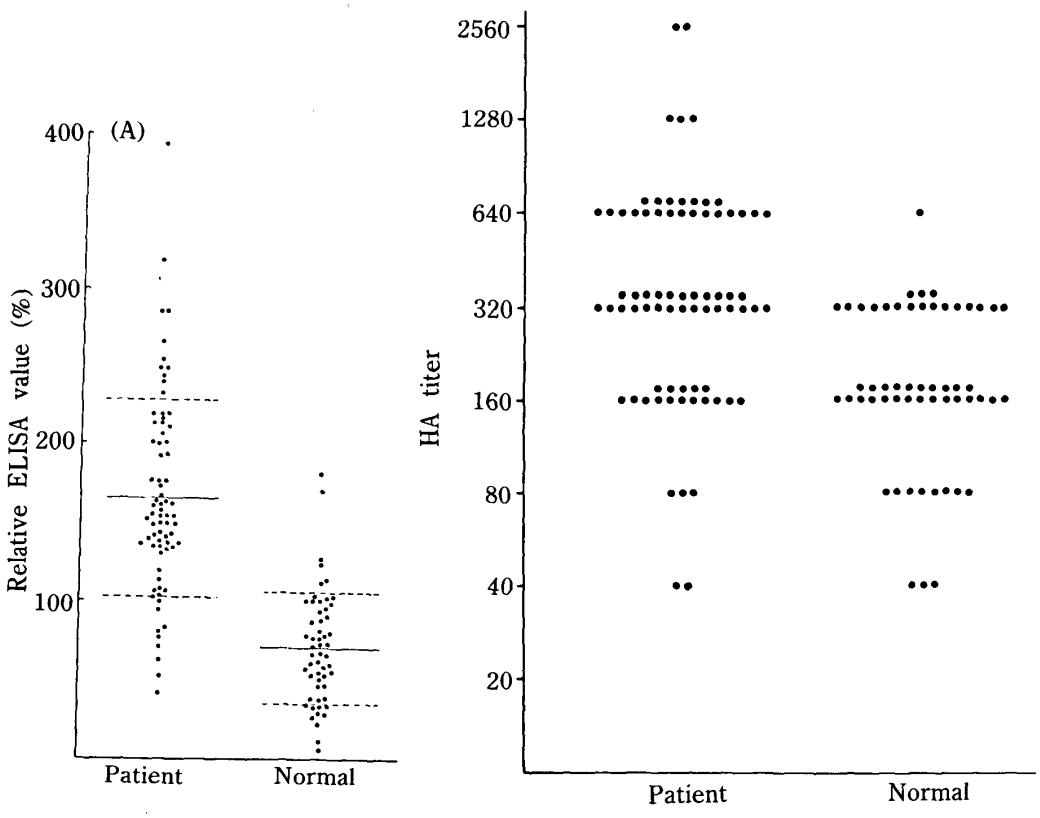

FIG. 1 Anti-CPH ELISA values and rabbit erythrocyte hemagglutinin (HA) levels in sera from healthy persons (Normal) and from H-D antibody-positive patients with various diseases (Patient). Anti-CPH ELISA values were determined with enzyme-labelled specific anti-human IgG (Fab) as the 2nd antibody, and each value represented an average of four determinations. The mean (——) and standard deviation (---) were calculated from the data.

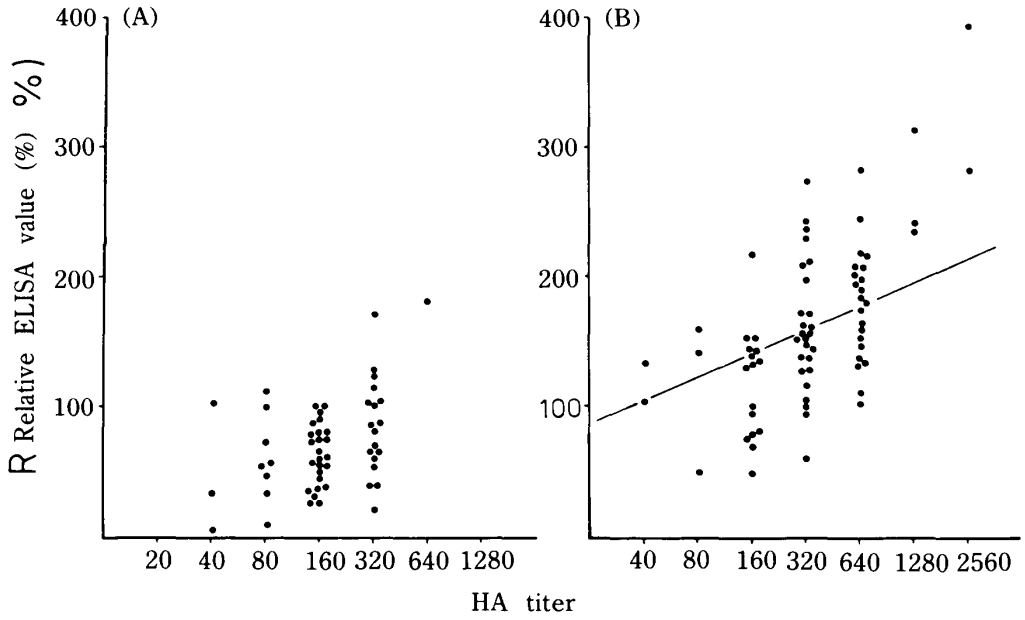

FIG. 2 Correlation between anti-CPH ELISA values and rabbit erythrocyte hemagglutinin (HA) titers in sera from healthy persons (A) and patients (B). Correlation coefficients (γ) in (A) and (B) were calculated as 0.0043 and 0.29, respectively. Only the latter one was significant ($p<0.05$).

TABLE 1 IgG and IgM antibody levels in sera which showed low or high ELISA values and low or high hemagglutinin (HA) titers

Serum	ELISA[1]	HA[2]	Antibody Levels[3]	
Donor	Value		IgG	IgM
Standard[4]	(100)	(160)	100	100
Normal A	Low (6)	Low (40)	11	114
Normal B	Low (21)	High (320)	16	186
Normal C	High (102)	Low (40)	153	79
Patient MN	High (134)	Low (40)	129	86
Patient NT	High (248)	High (1,280)	194	117
Patient MH	High (210)	High (640)	86	217

[1] The levels of antibodies to CPH were determined by ELISA using specific anti-IgG (Fab) as shown in Fig. 1 .

[2] The hemagglutination test with rabbit erythrocytes was performed as shown in Fig. 1 .

[3] The levels of IgG and IgM antibodies to CPH were titrated by ELISA using specific anti-IgG (γ) and anti-IgM (μ) as the 2nd antibody, respectively. The antibody levels were calculated from the integrating area under the titration curve as described in MATERIALS AND METHODS.

[4] The serum (E.S.) was usually used as a standard serum for calculation of a relative ELISA value as described in MATERIALS AND METHODS.

levels are higher in patient sera (FIGS. 1 & 2).

The H-D antibody-positive sera were divided into three groups: sera from patients with tumors, infectious diseases and other diseases by clinical diagnosis. Each group of sera showed significantly higher levels of anti-CPH antibody than those of normal sera (FIG. 3).

The anti-CPH IgG antibody levels in other cancer patients randomly selected were examined. In 4 out of 67 cancer patients examined (two with pancreatic cancer, one with hepatoma and one with gastric cancer), apparently higher levels of anti-CPH antibody over the normal range were detected, although the average of antibody levels in this group was lower that in the H-D antibody-positive patients (FIG. 4).

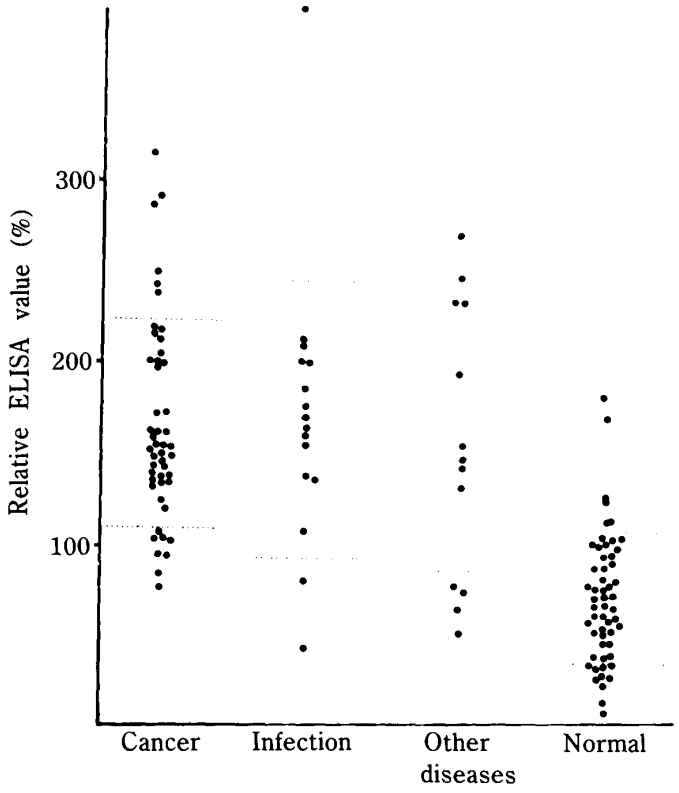

FIG. 3 Comparison of anti-CPH ELISA values in sera from healthy persons (Normal) and from H-D antibody-positive patients with cancer, infectious diseases or other diseases. ELISA values of patients shown in Fig. 1 (A) were classified into three groups according to their respective diseases.

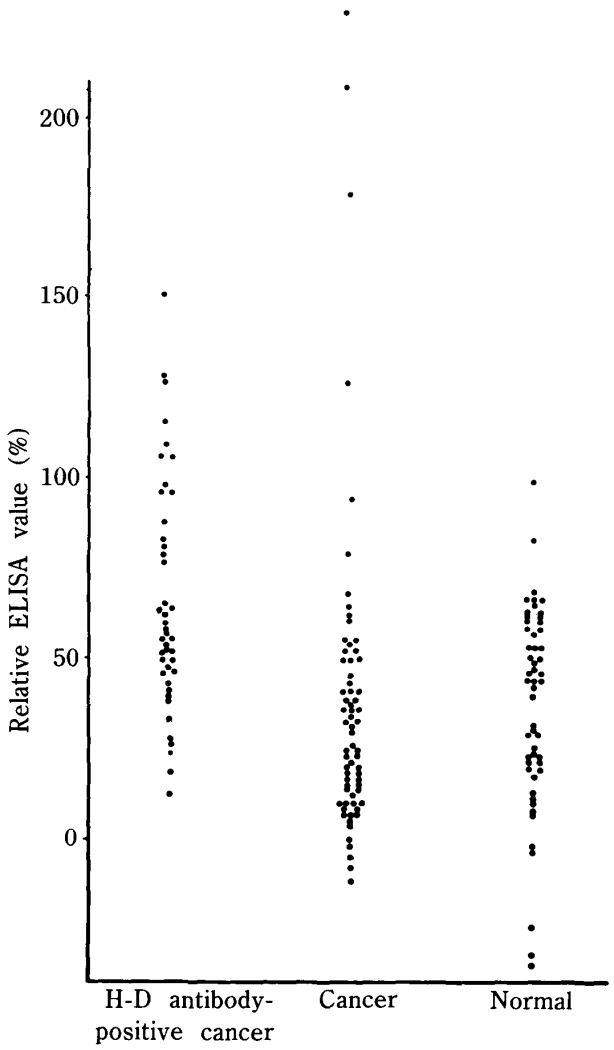

FIG. 4 Comparison of anti-CPH ELISA values in sera from healthy persons (Normal), H-D antibody-positive cancer patients and randomly selected cancer patients. The antibody levels were determind by ELISA using specific anti-IgG (γ).

Discussion

Tönder et al.[15] compared the rabbit erythrocyte hemagglutinin levels in normal sera from 880 healthy persons with those in pathologic sera from 345 patients and found no distinctly different distribution between the two groups, except abnormally high titers in sera from 7 patients with chronic cold hemagglutinin disease or rheumatoid arthritis, and abnormally low titers in sera from 5 patients with agammaglobulinemia. In the present studies, significantly high levels of heterophilic antibody were observed in patient sera by both the hemagglutination test and ELISA (Fig. 1). The discrepancy between the two results might have occurred because H-D antibody-positive sera screened from many patient sera by the hemagglutination test were used in this experiment. In fact, when randomly selected cancer patient sera were used, the mean value of antibody levels detected ELISA was not higher than that of normal sera, although abnormally high levels of the antibody were detected in several individual sera (Fig. 4). These results suggest that these two heterophilic antibodies are elevated under similar conditions. However, correlation between anti-CPH and anti-hematoside (NeuGc) antibody levels detected by ELISA was not significant (Suzuki et al., unpublished). The expression of H-D antigen in cancerous tissues was already demonstrated,[4,5,9,10] and similarly, CPH may be expressed. But the two antigens may be expressed differntly in amounts in tumor cells in each patient. Or, it is possible that the two antibody productions are stimulated by different mechanisms. Recently, it was found that the natural anti-CPH antibodies in human sera play a physiological role to remove senescent erythrocytes from the blood stream.[2] The CPH antigen, or a cross-reacting antigen, may be exposed on senescent erythrocytes, though such a glycosphingolipid has not been detected by any researchers so far, and it was found that the natural antibodies bind to the old erythrocytes to be removed by the antibody-dependent cytolytic reaction of monocytes.[3] If antigen-exposing cells such as senescent erythrocytes appear under some pathologic condition, the CPH antibody production may be stimulated. In order to understand the role of anti-CPH antibody and the the mechanism of its elevation in patients, the antigen expression in various tissues must be examined.

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