BIKEN JOURNAL Vol. 24 23-37, 1981

HANGANUTZIU AND DEICHER TYPE HETEROPHILE ANTIGEN EXPRESSED ON THE CELL SURFACE OF MAREK'S DISEASE LYMPHOMA-DERIVED CELL LINES

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SUMMARY Antibodies to Marek's disease (MD) tumor-associated surface antigen (MATSA) were prepared by immunizing chickens with the MSB1 lymphoblastoid cell line derived from an MD lymphoma. All the chicken anti-MSB1 sera examined contained heterophile antibodies, which agglutinated sheep red blood cells (SRBC) and were absorbed with bovine red blood cells (BRBC) or the precipitate of a guinea pig kidney homogenate. Their heterophile properties were identical with those of Hanganutziu and Deicher (H-D) heterophile antibodies in humans. The heterophile antibodies were also stimulated in chickens, even when the MSB1 cells cultured in medium containing homologous chicken serum instead of the heterologous fetal calf serum were used for immunization. H-D antigenic molecules were previously purified from equine and bovine erythrocytes as the hematoside N-glycolylneuraminyl lactosyl ceramide and ganglioside N-glycolylneuraminyl lactneotetraosyl ceramide, respectively. Both glycosphingolipids specifically diminished the heterophile activity of chicken anti-MSB1, but did not completely inhibit MATSA activity. For demonstration of the specific expression of H-D antigen on MD tumor-derived cell lines, the antibodies to the two glycosphingolipid antigens and SRBC were prepared in chickens by immunization. The antibodies of anti-hematoside and anti-SRBC were purified with BRBC stroma as immunoadsorbent. These antisera and the purified antibodies cross-reacted with both the glycosphingolipids, but not with another ganglioside (GM_1) which has a different carbohydrate structure, in immunodiffusion and hemagglutination inhibition tests. The presence of H-D antigen on MD lymphoma-derived cell lines MSB1 and HP1 was demonstrated using these antisera and purified antibodies by the indirect and/or direct membrane immunofluorescence technique and the complement-dependent antibody cytotoxicity test. However, weak or negative reactions were observed with the transplantable MD tumor-derived cell lines RP1 and BP1, and the avian lymphoid leukosis lymphoma-derived cell lines 1104B1 and 1104X5. These results suggest

that so-called MATSA, recognized by chicken anti-MSB1 sera, consists of at least two different antigens: one is H-D antigen and the other has an unknown chemical nature.

INTRODUCTION

Marek's disease (MD) is an extensively studied lymphoproliferative disease of chickens caused by a herpesvirus named MD virus (MDV). Since an MD lymphoma-derived cell line was first established by Akiyama et al. (1973), many cell lines derived from MD lymphomas or transplantable MD tumors have been established, and these cell lines have greatly facilitated studies on MD tumor cells. MD lymphoma-derived cell lines, such as MSB1 (Akiyama and Kato, 1974) and HP1 (Powell et al., 1974), carry a T-cell surface antigenic marker (Nazerian and Sharma, 1975; Matsuda et al., 1976a) and an MD tumor-associated surface antigen (MATSA) (Powell et al., 1974; Witter et al., 1975; Matsuda et al., 1976b). However, virus-specific intracellular and membrane antigens have been detected on only a small percentage (less than 1%) of the cells. Some cell lines, such as RP1 (Nazerian et al., 1977) and BP1 (Sekiya et al., 1977), which were derived from a transplantable MD tumor induced by an oncogenic JM strain of MDV (Sevoian et al., 1964), also carry a T-cell marker and MATSA, but no virus-specific intracellular or membrane antigens.

MATSA was not detected on normal chicken lymphocytes, lymphoma cells of avian lymphoid leukosis (LL), or MDV-infected fibroblasts (Witter et al., 1975). Furthermore, this antigen was unrelated to embryonic antigen (Witter et al., 1975; Murthy et al., 1979) or to histocompatibility antigen (Witter et al., 1975). MATSA is not identical in different MD cell lines: dissimilarities in the antigen in different cell lines have been observed by several workers (Witter et al., 1975; Nazerian et al., 1977; Sharma et al., 1977; Sugimoto et al., 1979b; Rennie and Powell, 1979).

On the other hand, the expression of heterophile antigens has been demonstrated on some transformed human cells. Forssman glycosphingolipid antigen was isolated from human gastric cancer tissues as blood group A-like neoantigen by Hakomori et al. (1977). Paul and Bunnell (P-B) antigen was demonstrated on the cell membrane or surface of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (Shope and Miller, 1973; Maeda et al., 1979) and P-B antibodies can be detected in sera of almost all patients with infectious mononucleosis, the etiological agent of which is considered to be EBV. Nishimaki et al. (1979) demonstrated Hanganutziu and Deicher (H-D) antigen in sera and malignant tissue extracts from patients with various types of cancer.

Among these three heterophile antigens, only H-D antigen is known to be heterophilic in chickens and little is known about P-B antigen. The H-D antigenic determinant requires N-glycolylneuraminic acid at the nonreducing end of the carbohydrate chain of complex carbohydrates (Higashi et al., 1977), but chickens cannot synthesize this kind of sialic acid (Klenk and Uhlenbruck, 1958). Therefore, in preliminary experiments we examined the relation of H-D antigen to MATSA and found that chicken anti-MSB1 sera with anti-MATSA specificity also contained a heterophile antibody with H-D-like antibody specificity (Ikuta et al., 1980). This communication reports evidence for the presence of H-D antigen on MD lymphoma-derived cell lines, and describes the relation of this antigen to MATSA.

MATERIALS AND METHODS

1. *Cells*

The avian lymphoblastoid cell lines used were

MDCC-MSB1 (Akiyama and Kato, 1974), -HP1 (Powell et al., 1974), -RP1 (Nazerian et al., 1977), -BP1 (Sekiya et al., 1977), LSCC-1104B1 and -1104X5 (Hihara et al., 1974). These cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum or in RPMI-1640 supplemented with 2% chicken serum at 41 C in a humidified atmosphere of 5% CO_2 in air. HP1, RP1, BP1 and two LL lymphoma-derived cell lines were kindly supplied by Dr. Powell, Houghton Poultry Research Station, England, Dr. Nazerian, Regional Poultry Research Laboratory, USA, Dr. Sekiya, Bio Pharmaceuticals, Inc., Japan, and Dr. Hihara, National Institute of Animal Health, Japan, respectively.

2. *Purification of H-D antigens*

The H-D antigen-active glycosphingolipids hematoside and ganglioside were purified from equine and bovine erythrocyte stroma, respectively (Higashi et al., 1977). GM_1 ganglioside was purified from bovine brain as described previously (Naiki et al., 1974) and used as an H-D antigen-negative compound. The structures of these glycosphingolipids are given in Table 1.

3. *Preparation of antisera against H-D antigen*

Specific pathogen-free chickens of more than 6 months old were immunized intramuscularly (i.m.) with 1 mg of equine hematoside or bovine ganglioside mixed with an equal amount of methylated bovine serum albumin and complete Freund's adjuvant. After four weeks, the chickens were given a booster injection of 2 mg of antigen in the same way, and were bled 7 to 10 days after the booster injection. Antiserum against sheep red blood cells (SRBC) was also prepared by i.m. immunization of chickens of the same age with 1×10^9 SRBC mixed with complete Freund's adjuvant, and from two weeks later, with several intravenous (i.v.) injections of 1×10^8 to 5×10^8 SRBC at 7-day intervals.

4. *Purification of H-D antibodies*

Antibodies were purified from chicken anti-hematoside and chicken anti-SRBC sera. Samples of 2 ml of each two-fold diluted antiserum were incubated for 1 h at 37 C with shaking with 50 mg of hovine red blood cell (BRBC) stroma (Difco Laboratories), which had been washed once with 0.12 *M* citrate buffer, pH 3.0, and three times with a large volume of phosphate-buffered saline, pH 7.4 (PBS). Then, the insoluble cell material adsorbing antibodies was washed five times with a large volume of PBS, suspended in 2 ml of 0.12 *M* citrate buffer, pH 3.0, incubated for 20 min at 37 C with shaking, and centrifuged. The resulting supernatant containing released antibodies was mixed with 1 ml of 1 *M* tris-HCl, pH 9.0, and the antibodies were precipitated by addition of 3 ml of saturated ammonium sulfate. The precipitate was dissolved in 0.1–0.5 ml of PBS and dialyzed against PBS.

5. *Preparation of antisera against MATSA*

Anti-MATSA sera were prepared by immunizing eight specific pathogen-free chickens of over 6 months old (lots 1 to 8) with fixed or unfixed MSB1 cells. Lot 1 serum was ohtained by i.v. immunization with 1×10^8 cells fixed with periodate-lysine-paraformaldehyde (PLP) as described previously (Matsuda et al., 1976b). Lot 2 serum was obtained by immunization with 1×10^9 of PLP-fixed cells mixed with incomplete Freund's adjuvant, and then a booster i.v. injection of 1×10^8 of the

TABLE 1. *Structures of glycosphingolipids used*

Glycosphingolipid	Chemical structure
Equine hematoside	NeuGc (α, 2-3) Gal (β, 1-4) Glc-Cer
Bovine ganglioside	Gal (β, 1-4) GlcNAc (β, 1-3) Gal (β, 1-4) Glc-Cer | (α, 2-3) NeuGc
GM_1 ganglioside	Gal (β, 1-3) GalNAc (β, 1-4) Gal (β, 1-4) Glc-Cer | (α, 2-3) AcNeu

Abbreviations: Gal, D-galactose; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; NeuGc, N-glycolylneuraminic acid; AcNeu, N-acetylneuraminic acid; Cer, ceramide.

same cells. Lot 3 serum was obtained by i.m. immunization with 1.5×10^7 cells fixed with 0.125% glutaraldehyde, and then a booster i.v. injection of 1×10^6 of the same cells. Lots 4 to 6 sera were obtained by i.v. immunization with 1×10^8 unfixed cells. Lots 7 and 8 sera were obtained by i.m. immunization with 1×10^7 unfixed cells mixed with complete Freund's adjuvant, and then a booster i.v. injection of 1×10^7 to 10^8 cells. For preparation of lots 1 to 6 of sera, MSB1 cells cultured in RPMI-1640 supplemented with 10% fetal calf serum were used, while for preparation of lots 7 and 8 of sera, MSB1 cells cultured in RPMI-1640 supplemented with 2% chicken serum for more than 4 months were used. Of these anti-MSB1 sera, lots 3–6 were kindly supplied by Dr. T. Mikami, Hokkaido University, Japan.

6. *Immunodiffusion test*

The immunodiffusion test was performed in 1% agarose in PBS containing 8% NaCl. Circular wells, 5 mm in diameter, were cut at an edge-to-edge distance of 2.5 mm.

7. *Titration of heterophile antibody*

Agglutination titers were assayed using a microplate. Serial two-fold dilutions of antiserum (25 μl) were mixed with 0.5% SRBC suspension (25 μl) and kept at room temperature for 3 h before reading the end point of complete agglutination. PBS was used as buffer solution for dilution. Titers are shown as reciprocals of the end point.

8. *Membrane immunofluorescence (MIF) test*

The indirect MIF test was performed as described by Witter et al. (1975) with slight modifications. Briefly, about 2×10^6 cells washed with PBS were mixed with 25 μl of serial dilutions of antiserum and 25 μl of PBS, and then incubated for 30 min at 37 C. Then the cells were washed twice with a large volume of PBS, and incubated with 25 μl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG serum for 30 min at 37 C. The cells were again washed twice with a large volume of PBS, and then a drop of cell suspension was placed on a coverslip and promptly examined with a fluorescent microscope.

For the direct MIF test, H-D antibody purified from chicken anti-hematoside was conjugated with FITC as described previously (Naito et al., 1969). About 2×10^6 cells were mixed with 25 μl of this FITC-conjugated antibody and 25 μl of PBS and incubated for 1 h at 37 C. Then the cells were washed twice with a large volume of PBS and the number of fluorescence-positive cells was counted in the same way.

9. *Complement-dependent antibody cytotoxicity (CDAC) test*

Chicken antibody-antigen complex cannot bind with guinea pig complement, C'1, but can bind duck complement (Benson et al., 1961; Fukuda, 1971). Thus, Sugimoto et al. (1979a) succeeded in examining the cytotoxicity of MSB1 cells using chicken anti-MSB1 serum with duck complement. In this work the CDAC test was carried out using duck complement as follows: volumes of 25 μl of line cells (5×10^6 cells/ml) were mixed with 25 μl of serial dilutions of antiserum or antibody solution and 25 μl of 5-fold diluted duck complement in a microplate, and incubated for 1 h at 37 C. The number of viable cells was then counted by trypan blue dye exclusion. As a control, a 4-fold dilution of normal chicken serum was used in place of antiserum. The cytotoxic index (CI) was calculated by the following formula:

$$\mathrm{CI}(\%) = \frac{\text{Number of viable cells in the control} - \text{Number of viable cells in the test sample}}{\text{Number of viable cells in the control}} \times 100$$

10. *Inhibition test*

Inhibition tests of SRBC agglutination and MIF, were carried out by addition of equine hematoside, bovine ganglioside or GM_1 ganglioside (10, 100 or 500 μg/ml in PBS containig 0.05% sodium taurodeoxycholic acid), as described previously (Naiki and Marcus, 1974). These inhibitors (25 μl) were added to serial dilutions of antiserum (25 μl), and mixed with SRBC or avian cells, and tested out as described above.

11. *Absorption test*

Guinea pig kidney was homogenized, boiled, and centrifuged. The precipitate (GPKP) was washed three times with a large volume of PBS and used for absorption. SRBC, BRBC or chicken red blood cells (CRBC) were also used after being washed with PBS. Two-fold diluted antiserum was mixed with half volumes of each of these absorbent cells and kidney precipitate. The mixtures were incubated for 30 min at 37 C, and then centrifuged

at 4,000 *g* for 5 min. The supernatant was again mixed with a half volume of the same absorbent cells and similarly incubated. This absorption was repeated three times, and then the supernatant was recovered.

RESULTS

1. *Heterophile properties of chicken anti-MSB1 sera*

Chicken anti-MSB1 sera, lot 1 to 6, contained various levels of SRBC agglutinin as well as anti-MATSA activity against MSB1 cells (Tabel 2). SRBC agglutinin and anti-MATSA activities were also detected in immune sera (lots 7 and 8) obtained by immunization with MSB1 cells cultured in medium supplemented with homologous chicken serum in place of fetal calf serum containing heterophile antigen. The results of SRBC agglutination titers were not always proportional to the MATSA titers. No SRBC agglutinin activity was detectable in sera from 30 specific pathogen-free chickens of various ages. The heterophile antibodies in anti-MSB1 sera (lots 1 and 7) were absorbed with cells from foreign species, SRBC, BRBC and GPKP, by the method of Davidsohn (1938) (Table 3).

TABLE 2. *Presence of heterophile antibodies in chicken anti-MSB1 sera*

Antiserum		SRBC agglutination titer	MATSA titer[a]
Anti-MSB1[b]	1	64	320
	2	4	40
	3	4	160
	4	64	320
	5	4	40
	6	8	40
	7	8	80
	8	8	80
Normal chicken serum[c]		<2	<2

[a] The highest dilution giving a positive reaction in more than 20% of MSB1 by the indirect MIF technique.

[b] Lots 1 to 3 were anti-fixed cells, and lots 4 to 8 were anti-intact cells. The cells were grown with fetal calf serum for preparation of lots 1 to 6 and with chicken serum for preparation of lots 7 to 8.

[c] All sera from twenty chickens of one day old, five chickens of 3 months old, and five chickens of 6 months old gave the same results.

TABLE 3. *Absorption test of chicken anti-MSB1 with various cells*

Anti-MSB1	Absorption with	SRBC agglutination titer	MATSA titer[a]
Lot 1[b]	None	64	320
	GPKP[c]	<2	20
	BRBC[d]	<2	20
	SRBC[e]	<2	20
	CRBC[f]	32	160
Lot 7[b]	None	8	80
	GPKP	<2	20
	BRBC	<2	20

[a] See Table 2.

[b] Lots 1 and 7 were the same antisera as for Table 2.

[c] Guinea pig kidney precipitate.

[d] Bovine red blood cells.

[e] Sheep red blood cells.

[f] Chicken red blood cells.

The reactivities of both antisera with SRBC were completely eliminated by SRBC, BRBC and GPKP, but not CRBC. The properties of the heterophile antibody in chicken anti-MSB1 serum were identical with those of H-D antibody, but not with those of Forssman or P-B antibody in human sera. On the other hand, the reactivities of anti-MSB1 serum (lot 1) with the cell surface of MSB1 were considerably reduced by absorption with SRBC, BRBC or GPKP, but not CRBC. The SRBC agglutination of anti-MSB1 (lot 1) was inhibited by addition of H-D antigen-active glycosphingolipids, ganglioside or hematoside, but not by addition of GM_1 ganglioside, and the number of fluorescence-positive MSB1 cells was also decreased by addition of H-D ganglioside, but not of GM_1 ganglioside (Table 4).

TABLE 4. *Test of inhibition by glycosphingolipids of chicken anti-MSB1 (lot 1) in SRBC agglutination and indirect MIF tests*

Inhibitor[a]	SRBC agglutination titer	MIF test[b] Dilution of anti-MSB1			
		×8	×32	×128	×512
None	32	>90[c]	>90	70	20
Bovine ganglioside	8	80	60	50	0
Equine hematoside	8	ND[d]	ND	ND	ND
GM_1 ganglioside	32	>90	>90	50	20

[a] SRBC agglutination and indirect MIF tests were performed in the presence of 25 μl of each glycosphingolipid (500 μg/ml).
[b] The MIF test was performed using MSB1 line cells, which were cultured in medium supplemented with chicken serum.
[c] The percentage of fluorescence-positive cells is shown.
[d] Not done.

TABLE 5. *Indirect MIF test on chicken anti-MSB1 (lot 1) absorbed three times with SRBC*

Cells	Cultured with	Dilution of antiserum				
		×2	×4	×8	×16	×32
MSB1	FCS[b]	>90[d]	>90	>90	90	50
	CHS[c]	>90	>90	>90	>90	90
HP1	FCS	>90	>90	>90	60	20
	CHS	>90	>90	>90	>90	70
BP1	FCS	>90	>90	>90	50	20
	CHS	>90	>90	>90	90	70
1104B1	FCS	<10	<10	0	0	0
NCSL[a]		<10	<10	0	0	0

[a] Normal chicken splenic lymphocytes.
[b] Fetal calf serum.
[c] Chicken serum.
[d] The percentage of fluorescence-positive cells is shown.

The above results suggests that H-D antigen is one, but not the only, component of so-called MATSA. In fact, when one chicken anti-MSB1 serum (lot 1) had been absorbed three times with SRBC, it still reacted with MSB1, HP1 and BP1 cells, which were derived from MD tumors, but not with 1104B1 cells derived from an LL lymphoma (Table 5). The absorbed serum also did not react with normal splenic lymphocytes from several chickens. These results suggest that the remaining antibody also has the properties of MATSA.

2. *Specificity of immune sera and purified antibodies*

To demonstrate the presence of H-D heterophile antigen on MD lymphoid cells more clearly, we prepared antibodies to H-D antigen-active glycosphingolipids in chickens by

the immunization procedure. Anti-equine hematoside serum and anti-bovine ganglioside serum both gave a specific precipitin band against both compounds, but not against GM_1 ganglioside on double diffusion in agarose gel (Fig. 1a). These precipitin bands were clearly detectable even 6 h after addition of the antisera and the antigens. The precipitin band observed between hematoside and the homologous antiserum and/or between gan-

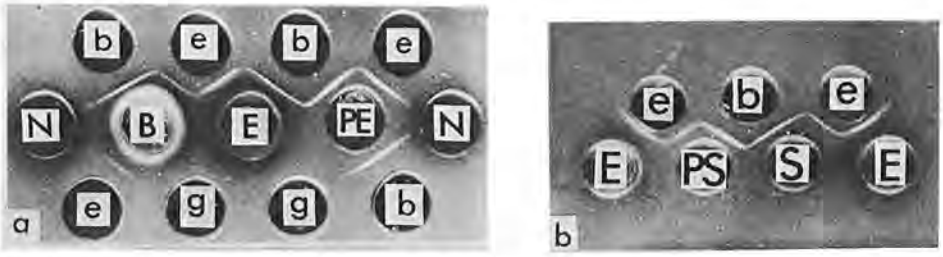

FIGURE 1. Immunodiffusion test on chicken antisera and purified antibodies preparations. b, bovine ganglioside; e, equine hematoside; g, GM_1 ganglioside; B, anti-bovine ganglioside; E, anti-equine hematoside; PE, antibody purified from anti-equine hematoside; S, anti-SRBC; PS, antibody purified from anti-SRBC; N, normal chicken serum. Each glycosphingolipid was dissolved in PBS containing 0.05% sodium taurodeoxycholic acid at a concentration of 50 μg/ml, and used as antigen. The procedures for the immunodiffusion test and purification of the antibodies are described in the Materials and Methods.

TABLE 6. *Inhibition and absorption tests of chicken antisera or purified antibodies to H-D antigen-active glycosphingolipids and SRBC*

Treatment		SRBC agglutination titer				
		Anti-equine hematoside	Anti-bovine ganglioside	Anti-SRBC	Antibody purified from anti-equine hematoside	Antibody purified from anti-SRBC
None		512	256	128	8192	1024
Inhibition[a]						
with equine hematoside	10	32	128	ND[d]	ND	ND
	100	16	64	ND	ND	ND
	500	8	32	ND	128	16
with bovine ganglioside	10	32	64	ND	ND	ND
	100	16	64	ND	ND	ND
	500	8	16	ND	128	16
with GM_1 ganglioside	500	512	256	ND	8192	1024
Absorption						
with GPKP[b]		2	8	4	<2	<2
with BRBC[c]		<2	<2	<2	2	<2

[a] The test of inhibition of SRBC agglutination was performed by addition of 25 μl of each glycosphingolipid (10, 100 or 500 μg/ml).
[b] Guinea pig kidney precipitate.
[c] Bovine red blood cells.
[d] Not done.

glioside and the homologous antiserum appeared stronger than those between either of these two compounds and antisera to the other compounds. However, these complete fusions may be spurious cross-reactions as indicated by Naiki et al. (1974). Therefore, the cross-reactivity was confirmed by inhibition tests of SRBC hemagglutination (Table 6). Both the antisera strongly hemagglutinated SRBC and their titers decreased with increase in the dose of hematoside or ganglioside, but were not affected by addition of GM_1 ganglioside even at the highest dose tested (500 μg/ml). Anti-hematoside serum and the antibody purified from the antiserum were inhibited equally by hematoside and ganglioside, but anti-ganglioside serum was inhibited more by ganglioside than by hematoside. Details of immunochemical studies on the specificities of the two antisera will be reported in the near future.

Chicken anti-SRBC serum should also have H-D antibodies. Therefore, we purified heterophile antibodies from the serum with BRBC stroma as immunoadsorbent. The purified anti-SRBC antibody did indeed give a precipitin line with both hematoside and ganglioside in the gel diffusion test (Fig. 1b), and its hemagglutination was strongly inhibited by both compounds, but not by GM_1 ganglioside (Table 6). The antisera and purified antibodies showed the clearest H-D antibody-positive reactions in Davidsohn's absorption test; that is, almost all the hemagglutinin to SRBC was absorbed with GPKP or BRBC (Table 6).

3. *Demonstration of H-D antigen on MD cell lines*

Before examining the expression of H-D antigen on various avian lymphoblastoid cell lines, these lines were cultured in medium supplemented with chicken serum for more than 4 months to remove H-D antigen-active substances present in fetal calf serum, such as fetuin (Callahan, 1976), as completely as possible. Before examination, the BRBC, which should have H-D antigen, and CRBC, which do not have the antigen, were stained with anti-hematoside serum and the purified antibody of anti-SRBC by the indirect MIF technique. BRBC gave positive reactions, but CRBC gave negative reactions with both antibody preparations. Positive cells were seen as a ring. The number of positive BRBC decreased at the optimal dilution of antibodies in the presence of H-D antigen-active glycosphingolipids as inhibitors, but the inhibitory activity of the glycosphingolipids was weaker at a higher concentration of the an-

TABLE 7. *Inhibition test on the indirect MIF test using bovine red blood cells*

Cells	Inhibitor[a]	Dilution of chicken anti-equine hematoside						Dilution of antibody purified from chicken anti-SRBC[b]			
		×4	×16	×64	×256	×1024	×4096	×4	×8	×16	×32
BRBC[c]	None	>90[e]	>90	>90	80	10	<10	>90	80	30	10
	Equine hematoside	>90	80	50	<10	0	0	80	40	20	0
	Bovine ganglioside	>90	80	40	<10	0	0	80	40	20	0
CRBC[d]	None	0	0	0	0	0	0	0	0	0	0

[a] The indirect MIF test was performed as described in the Materials and Methods in the absence and presence of 25 μl of equine hematoside or bovine ganglioside (500 μg/ml).
[b] See Table 6.
[c] Bovine red blood cells.
[d] Chicken red blood cells.
[e] The percentage of fluorescence-positive cells is shown.

TABLE 8. *Indirect and direct MIF tests with chicken anti-equine hematoside*

Cells	Inhibitor[a]	Indirect MIF test[b]						Direct MIF test[b]
		Dilution of antiserum						
		×4	×16	×64	×256	×1024	×4096	
MSB1	−	90[d]	90	70	10	<10	0	38.9
	+	90	80	50	<10	0	0	8.8
HP1	−	80	80	60	10	<10	0	31.2
	+	70	80	30	0	0	0	6.9
RP1	−	20	<10	0	0	0	0	1.9
	+	20	<10	0	0	0	0	ND[e]
BP1	−	<10	0	0	0	0	0	0
	+	<10	0	0	0	0	0	ND
1104B1	−	90	90	40	<10	0	0	1.9
	+	90	90	30	0	0	0	1.3
1104X5	−	20	10	<10	0	0	0	0.7
	+	20	<10	0	0	0	0	ND
NCSL[c]	−	10	<10	0	0	0	0	0

[a] The MIF test was performed in the presence (+) and absence (−) of 25 μl of 500 μg/ml of equine hematoside.

[b] Chicken anti-equine hematoside serum was used in the indirect MIF test. Antibody purified from the antiserum was used in the direct MIF test after conjugation with FITC.

[c] Normal chicken splenic lymphocytes.

[d] The percentage of fluorescence-positive cells is shown.

[e] Not done.

tibodies (Table 7).

Table 8 shows the results of indirect and direct MIF test on avian lymphoblastoid cell lines using anti-hematoside serum and purified antibodies. High percentages (about 90%) of the MD lymphoma-derived line cells MSB1 and HP1 and one of the LL lymphoma-derived line cells, 1104B1, reacted with the antiserum. But smaller percentages (about 20%) of the transplantable MD tumor-derived line cells RP1 and BP1, and LL lymphoma-derived 1104X5 line cells, and also normal splenic lymphocytes reacted. However, the positive cells of LL lymphoma-derived 1104B1 cells were reduced in the direct MIF technique with purified antibody. In the direct method, only the MSB1 and HP1 cell lines gave positive reactions. The fluorescence was seen as patches on most cells, as shown in Fig. 2. Addition of hematoside at a concentration of 500 μg/ml slightly reduced the staining of positive cells by the indirect MIF with the antiserum, in the same way as with BRBC (Table 7), and greatly reduced the staining by direct MIF with purified antibody. The fact that MD lymphoma-derived line cells, but not transplantable MD tumor-derived line cells or LL lymphoma-derived line cells, gave a positive reaction with anti-hematoside was confirmed by CDAC tests using duck complement (Table 9).

Similar results were obtained in the indirect MIF test with anti-ganglioside serum, as shown in Table 10, although there were fewer fluorescence-positive cells than with anti-hematoside serum. Moreover, purified antibody

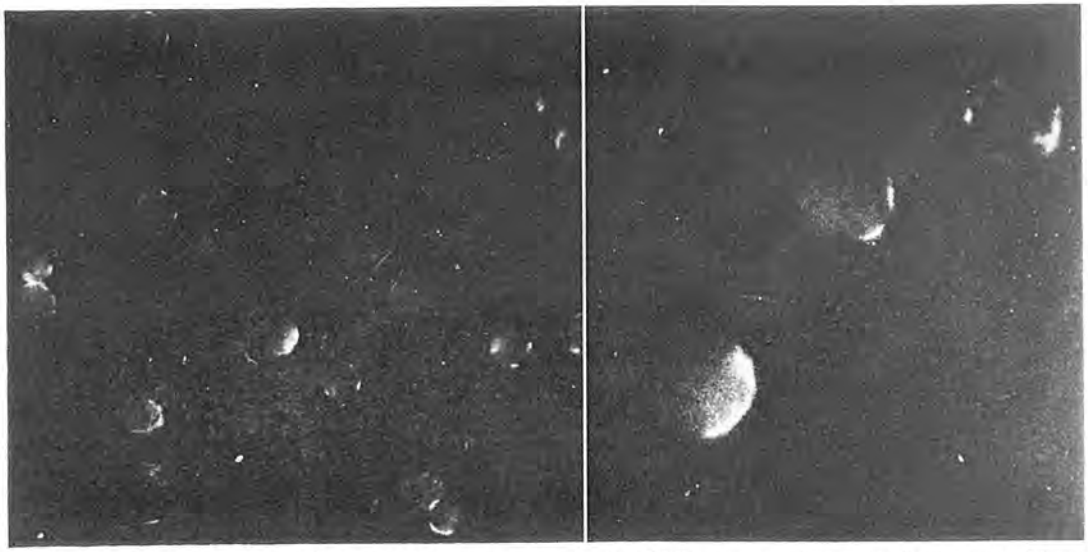

FIGURE 2. Fluorescent cells of the MSB1 cell line reacted with FITC-conjugated H-D antibody purified from chicken anti-equine hematoside in the MIF test. The direct MIF test was performed with FITC-conjugated antibody as described in the Materials and Methods. The right figure shows the same preparation at higher magnification.

TABLE 9. *CDAC test with chicken anti-equine hematoside and antibody purified from the antiserum*

Cells	Dilution of chicken anti-equine hematoside				Dilution of purified antibody			
	×4	×8	×16	×32	×2	×4	×8	×16
MSB1	31.8[a]	25.0	5.5	−6.5	28.8	16.5	16.1	−2.9
RP1	−2.3	−3.1	−3.9	0	−5.0	−8.7	−0.9	ND[b]
1104B1	−5.0	1.2	−5.0	1.9	2.5	1.7	0	ND

[a] The percentage CI is shown.
[b] Not done.

TABLE 10. *Indirect MIF test with chicken anti-bovine ganglioside*

Cells	Dilution of antiserum					
	×4	×16	×64	×256	×1024	×4096
MSB1	20[a]	40	30	10	<10	0
HP1	50	50	30	10	<10	0
RP1	<10	<10	0	0	0	0
BP1	<10	<10	0	0	0	0
1104B1	20	20	0	0	0	0
1104X5	10	10	0	0	0	0

[a] The percentage of fluorescence-positive cells is shown.

TABLE 11. *Indirect MIF test and CDAC test with H-D antibody purified from chicken anti-SRBC*

Cells	Inhibitor[a]	Indirect MIF test				CDAC test				
		Dilution of antibody				Dilution of antibody				
		×4	×8	×16	×32	×2	×4	×8	×16	×32
MSB1	−	80[b]	50	10	0	32.2[c]	29.9	17.3	13.8	1.2
	+	70	20	0	0	ND[d]	ND	ND	ND	ND
HP1	−	30	10	10	0	ND	ND	ND	ND	ND
	+	20	10	0	0	ND	ND	ND	ND	ND
RP1	−	0	0	0	0	12.5	−6.5	ND	ND	ND
BP1	−	0	0	0	0	ND	ND	ND	ND	ND
1104B1	−	10	0	0	0	7.9	−3.4	ND	ND	ND
1104X5	−	0	0	0	0	ND	ND	ND	ND	ND

[a] The indirect MIF test was performed in the presence (+) and absence (−) of 25 μl of 500 μg/ml of equine hematoside.
[b] The percentage of fluorescence-positive cells is shown.
[c] The percentage CI is shown.
[d] Not done.

of anti-SRBC serum also gave similar results, and distinguished the MD lymphoma-derived two cell lines from the other cell lines more clearly by the indirect MIF test than the CDAC test (Table 11).

DISCUSSION

MATSA has been demonstrated on MD lymphoma cells and MD lymphoma-derived cell lines with rabbit or chicken hyperimmune serum to them (Witter et al., 1975). However, the exact nature of this tumor antigen is not clearly understood. MATSA has been suggested to be a modified T-cell antigen (Ross et al., 1977) or a histocompatibility antigen (Bülow and Schmid, 1978), but there is some doubt about these suggestions (unpublished data, cited from Murthy et al., 1979).

We have given a preliminary report on the heterophile properties of MATSA (Ikuta et al., 1980), and more detailed data are presented in this paper. Anti-MSB1 sera containing anti-MATSA usually contain hemagglutinin activity to SRBC. This kind of hemagglutinin could not be absorbed with autologous CRBC, but was absorbed with heterologous materials, such as BRBC, SRBC and GPKP (Table 3). These heterophile properties prompted us to examine the possibility that H-D antigen is a component of MATSA.

On the other hand, Forssman antigen (Forssman, 1911) is one of the heterophile antigens in humans, but it is one of the autologous antigens in chickens. It is present in the vascular endothelium and the perivascular connective tissue (Tanaka and Reduc, 1956; Kitamoto et al., 1980). Recently, we examined the expression of Forssman antigen on various avian lymphoblastoid cell lines. The antigen was not detectable on cells from the MD lymphoma-derived cell lines MSB1 and HP1, but was detected on the transplantable MD tumor-derived cell lines RP1 and BP1, and on the LL lymphoma-derived cell lines 1104B1 and 1104X5 (Ikuta et al., 1981).

H-D antigen is present in the cells and tissue of many mammalian species, including

horse, cow, sheep, pig, goat, dog, cat, rabbit, guinea pig, mouse, hamster and monkey, but not in chicken and human tissues. H-D antigen was purified from equine and bovine erythrocytes as glycosphingolipids containing N-glycolylneuraminic acid; that is, as equine hematoside and bovine ganglioside, respectively (Higashi et al., 1977). But the antigenic activity was also detectable in a glycoprotein preparation from bovine erythrocytes (Naiki and Higashi, 1980), pig submaxillary gland mucin and a major glycoprotein, fetuin, of fetal calf serum (Callahan, 1976). Only H-D serum from patients has been used so far to examine the localization and expression of the antigen on cells or tissue sections (Kasukawa et al., 1976; Nishimaki et al., 1979). But human H-D serum is not ideal for this purpose, because few sera contain sufficient H-D antibody activity to give a precipitin reaction with H-D antigen-active substances, and different H-D sera have different affinities for H-D antigen-active compounds (Naiki and Higashi, 1980). Therefore, we tried to prepare a specific reagent for H-D antigen by immunizing chickens with H-D antigen-active glycosphingolipids and SRBC. Purification of H-D antibodies in anti-SRBC was necessary for this purpose, because SRBC have other antigens. Antisera to each glycosphingolipid and anti-SRBC serum reacted with equine hematoside, bovine ganglioside, BRBC glycoprotein and cat disialohematoside, NeuGc (α, 2–8) NeuGc (α, 2–3) Gal (β, 1–4) Glc-ceramide, as human H-D sera did (Fig. 1, Table 4 and data in preparation).

Using these antisera, we obtained evidence that H-D antigen is expressed on MD lymphoma-derived MSB1 and HP1 cell lines, but scarcely at all on transplantable two MD tumor-derived cell lines, two LL lymphoma-derived cell lines and/or normal chicken splenic lymphocytes. For demonstration of H-D antigen by the indirect MIF method, each cell preparation was carefully washed with a large volume of PBS until it gave a negative reaction with the second antibody (anti-chicken IgG), because the cells were cultured in the presence of chicken serum. But even after repeated washing the possibility of nonspecific reactions still remained, because it has been found that LL lymphoma-derived 1104B line cells have cell surface immunoglobulin (Oki and Hihara, 1976; Muto and Oki, 1977). One LL lymphoma-derived cell line, 1104B1, did indeed give a doubtful reaction by the indirect MIF technique using anti-hematoside serum (Table 8), but the erroneous reaction was clearly distinguished from the true one by the direct method using FITC-labeled purified antibody (Table 8). Moreover, antibody against H-D antigen-active glycosphingolipids is present in all chicken anti-MSB1 sera, because their SRBC hemagglutination activity was inhibited by these compounds (Table 4). However, the heterophile hemagglutinin titers in the sera were not always proportional to the staining titers to MSB1 cells in the indirect MIF test (Table 3), and some of the antibody population staining MSB1 remained after repeated absorption with SRBC, even though the agglutinin titer was completely removed (Table 3). The remaining antibody also stained all the other cell lines examined, except LL cell lines (Table 5). These results indicate that the component named MATSA by Witter et al. (1975) consists of H-D antigen and another antigen, which may be "genuine MATSA", but whose chemical nature is still unknown.

The RP1 and BP1 cell lines were derived from transplantable MD tumor JMV. This JMV kills chicks in 5 to 8 days, much less time than required for induction of tumors by inoculation of the virus or virus-producer cell lines such as MSB1. The RP1 and BP1 cell lines have a T-cell marker and MATSA, but lack any MDV rescuable in vivo or in vitro (Nazerian et al., 1977; Sekiya et al., 1977). On the other hand, the MSB1 and HP1 line cells, which are derived from MD lymphomas, also have a T-cell marker and MATSA, but produce MDV. In addition, qualitative analysis of MATSA in different MD cell lines has

shown a similarity in MSB1 and HP1, but a dissimilarity in JMV and MSB1 (Witter et al., 1975; Sharma et al., 1977; Nazerian et al., 1977; Sugimoto et al., 1979b; Rennie and Powell, 1979). The characteristics of naturally occurring MD lymphomas resemble those of MSB1 and HP1, but differ from those of RP1 and BP1. Previously, we showed a similarity of in vivo MD lymphoma cells to the MSB1 or HP1 cell line, but not to the RP1 or BP1 cell line in terms of Forssman antigenic expression (Ikuta et al., 1981). Preliminary examinations of expression of H-D antigen in in vivo spleen lesions have shown that the MD lymphoma cells so far examined were positive, while all the LL lymphoma cells so far examined were completely negative. These results also show a similarity of in vivo MD lymphoma cells to the MSB1 or HP1 cell line, but not to the RP1 or BP1 cell line in terms of H-D antigenic expression. N-Glycolylneuraminic acid is the immunodominant and essential group of H-D antigen. This kind of sialic acid can be synthesized by many mammalian species, but not by humans and chickens. N-Acetylneuraminic acid, but not N-glycolylneuraminic acid, was detected in sera (Mårtensson et al., 1958), red blood cells (Klenk and Lempfrid, 1957), kidney (Mårtensson et al., 1957), brain (Klenk and Uhlenbruck, 1958) and other organs of humans, and also in the sera and red blood cells of chickens (Klenk and Uhlenbruck, 1958). Both types of sialic acid have been found in many other mammalian species. Therefore, the expression of H-D antigen on line cells derived from MD lymphomas means that N-glycolylneuraminic acid-containing glycoconjugates should be present in the membranes of these cells. N-Glycolylneuraminic acid is synthesized from N-acetylneuraminic acid by an enzyme called "N-acetylneuraminate monooxygenase" (EC 1. 14. 99. 18) (Schauer, 1970). One possible explanation for the specific expression of H-D antigen on MD lymphoma-derived cell lines is that a gene coding for N-acetylneuraminate monooxygenase may be expressed in only part of the MD tumor population as a result of an interaction between the host gene and MDV gene and that MSB1 and HP1 cell lines are occasionally established from this part of the population. This is the first report of H-D antigen on chicken cells. We are now planning to demonstrate the actual presence of N-glycolylneuraminic acid and N-acetylneuraminate monooxygenase in MD lymphoma cells and MD lymphoma-derived cell lines.

ACKNOWLEDGMENTS

We are grateful to Dr. T. Amano of this Institute, Dr. Y. Hinuma, Kyoto University, Japan, and Dr. T. Sairenji, Kumamoto University, Japan, for valuable discussions. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and a grant from Mitsuhisa Cancer Foundation, Japan.

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