Cancer Immunol Immunother (1992) 35: 230–236

Cancer Immunology Immunotherapy

Detection of allogeneic Qa/TL and Ly specificities on murine tumor cells with IgD in tumor-regressor serum

Takae Tanino, Naohiro Seo, Takahiro Okazaki, Chiemi Nakanishi-Ito, Masayuki Sekimata, and Kohji Egawa

Department of Tumor Biology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo 108, Japan

Received 10 January 1992/Accepted 23 April 1992

Summary. Serum from C3H/He mice, which show regression of MM2 tumor cells after transplantation and removal (regressor serum, RS) contains non-gammaglobulin components that cross-react with various tumor cells of mice [22, 23]. In addition to tumor cells, various allogeneic lymphocytes are also susceptible to an RS-dependent lymphocyte-mediated cytotoxic reaction. To identify tumor cell surface antigens that cause the cross-reactive host response, the serum components were analyzed by absorption of RS with allogeneic lymphocytes. RS components were found to recognize allogeneic lymphocyte antigens including Qa-2 and Ly6.2. Specificity for the Qa-2 antigen was further tested using Qa-2-congenic mice. The expression of Qa-2 antigen was detected on the surfaces of MM2 and other tumor cells derived from H-2^k mice (seven among nine cell lines tested) by a membrane immunofluorescence method using a Qa-2-specific mAb. Physical characteristics of the Qa-2-specific component in RS were determined and found to differ from those of regular IgGs but to be similar to those of IgDs. Using an enzyme-linked immunosorbent assay with an IgD-specific mAb and Qa-2–lacZ fusion protein, the existence of IgD in RS with specificity for Qa-2 was confirmed. These results suggest that the RS component with Qa-2 specificity is an IgD, the specificity and physiological role of which are unknown.

Key words: Tumor antigen – Qa-2 – IgD

Introduction

Some established lines of transplantable ascites tumor cells of mice lose expression of H-2 antigens and thereby gain nonspecificity, becoming transplantable on allogeneic mice. It is presumed that these cells will be poor targets of H-2-restricted reactions by the hosts, but some cause strong reactions in syngeneic host mice even when the animals have not received specific immunization with the tumor antigens. These mice generally respond well to treatment by nonspecific immunopotentiating agents. In addition, the transplanted tumors sometime show regression after growth simply by removing most of the tumor cells. These properties have led to the proposal that such tumor cells strongly express cell-surface antigens that are targets of an H-2-nonrestricted response of the hosts. Although the biological properties of such H-2-deficient cell lines may not fully represent those of the primary tumor cells, they are nonetheless useful for obtaining information concerning cell-surface molecules responsible for H-2-nonrestricted immune reactions by the hosts.

The MM2 ascites tumor cell line, derived from a virally induced mammary tumor of C3H/He mice, is one such cell line. When MM2 cells are transplanted to the peritoneal cavities of C3H/He mice and are removed together with ascites after full growth of the ascitic cells, a considerable percentage of mice show complete regression of the tumor [8]. The regressor mice have random resistance against transplantation of MM2 and other tumor cells. When regressor mice are challenged with MM2 cells repeatedly they reject the inoculum and produce gammaglobulin antibody that inhibits transplantation of MM2 cells. The serum from the regressor mice (regressor serum, RS) obtained before the challenge also showed the ability to inhibit transplantation of the tumor cells [22]. Furthermore, RS can agglutinate a wide range of mouse ascites tumor cells [24] and lyse various tumor cells by a lymphocyte-mediated reaction [23]. All of these activities are mainly associated with non-gammaglobulin serum components, which so far are not clearly identified in RS. The wide target selectivity of the reactions is also enigmatic.

In the present study, the selectivity of the RS-dependent reactions is analyzed by identifying the tumor cell-surface substances that give rise to cross-reactive serum components in the host mice. The serum component that has binding specificity to one such cell-surface substance was partially characterized and found to be an IgD.

Correspondence to: K. Egawa

Table 1. H-2, Ly, Qa and TL phenotypes of various mouse strains used in this study

Strain	H-2	Ly-1	Ly-2	Ly-3	Ly-4	Ly-5	Ly-6	Ly-7	TL[a]	Qa-1	Qa-2	Qa-3
C3H/He	k	1	1	2	1	1	1	2	b	–	–	–
C3H-Ly6.2	k	1	1	2	1	1	2	2	b	–	–	–
CBA	k	1	1	2	1	1	1	2	b	–	–	–
CE	k	2	1	2	1	1	1	2	b	–	–	–
AKR	k	2	1	1	1	1	2	2	b	–	–	–
C58	k	2	1	1	1	1	2	1	a	+	–	–
BALB/c	d	2	2	2	1	1	1	2	c	–	–	–
DBA/2	d	1	1	2	1	1	2	2	c	–	+	+
DBA/1	q	1	1	2	1	1	1	2	b		+	–
A/J	a	2	2	2	1	1	1	2	a	+	+	+
C57BL/6	b	2	2	2	2	1	2	1	b	–	+	+
B6.K1	b	2	2	2	2	1	2	1	b	+	–	–
B6.K2	b	2	2	2	2	1	2	1	b	+	+	+

[a] TL^a, $1^+,2^+,3^+,5^+$; TL^b, $1^-,2^-,3^-,5^-$; TL^c, $1^-,2^+,3^-,5^-$

Materials and methods

Animals. Thirteen syngeneic mouse strains with known Qa, TL and Ly phenotypes (listed in Table 1) were used [9, 10]. B6, B6.K1 an B6.K2 are congenic with respect to the Qa-1, Qa-2 and Qa-3 antigens [21]. C3H/He and C3H-Ly6.2 are congenic regarding allotypes of the Ly6 antigen. The B6.K1, B6.K2 and C3H-Ly6.2 strains were generously provided by Dr. T. Takahashi of the Aichi Cancer Center Research Institute and maintained in our laboratory.

Tumor cells. MM2, MM46, MM48, MM102D, FM3A, MH134, MH125F, MH125P and X5563 cells were maintained by serial passages in the peritoneal cavities of C3H/He mice. MM46, MM48, MM102D, FM3A, MH125F and MH125P cells were kindly provided by Dr. H. Nagayama of the Research Institute for Tuberculosis and Cancer, Tohoku University. MM2, MM46, MM48 MM102D and FM3A cells were derived from virally induced mammary carcinoma of C3H/He mice. MH134, MH125F and MH125P were derived from chemically induced hepatomas of C3H/He mice. Meth A cells, derived from chemically induced hepatoma of BALB/c mice, were maintained also as ascites cells by passages in the syngeneic host mice. EL-4 cells derived from the B6 strain were maintained as ascites cells in B6 mice. BW5147 cells, derived from lymphoma of AKR mice, were maintained as an in vitro cell line.

Antibodies and complement. Qa-2-specific monoclonal antibody (mAb) of the IgG2b class (141-15.8) [16] was purchased from Australian Monoclonal Development, NSW. Rat mAbs specific to mouse IgG2b and to mouse IgD and the peroxidase derivative of the latter were purchased from the Meiji Institute of Health Science, Kanagawa, Japan. A mouse mAb (IgG2b class) specific to an anti-bacterial component (NusA protein of *E. coli*) was kindly provided by Dr. Y. Nakamura of this laboratory and was used as a mAb not related to tumor cell-surface antigens. $F(ab')_2$ of goat antibody specific to mouse immunoglobulin and its fluorescein isothiocyanate (FITC) derivative were from Tago, Inc., Burlingame, Calif. Fab′ of goat antibody specific to mouse immunoglobulin was prepared by reduction of the $F(ab')_2$ with 2-mercaptoethylamine and separation with Sephacryl S-200. Preabsorbed rabbit serum, used as a complement source, was from Ceder Lane Laboratories, Westbury, N. Y.

Regressor serum (RS). Regression of MM2 ascites tumor cells in C3H/He mice was induced as reported previously [8]. The regressor mice were bled by heart puncture under ether anesthesia. Serum (RS) was separated from the blood and stored at –60° C until used.

Adsorption of RS. For adsorption studies, RS was used after depletion of immunoglobulins by precipitation with ammonium sulfate at 0.35 saturation and then by starch-gel zone electrophoresis as described below. The resulting material was used as gammaglobulin-depleted RS although it may still contain some gammaglobulins. Gammaglobulin-depleted RS was diluted to 100 times the original volume of RS with RPMI-1640 medium supplemented with 7% fetal calf serum (FCS). A 1-ml sample of this solution was mixed with 2×10^7 lymphocytes (splenocytes or mixture of splenocytes and mesenteric lymph node cells), which had been freed of erythrocytes by the ammonium chloride method [1] and washed three times with Eagle's minimum essential medium (MEM), or with $(4-6) \times 10^6$ ascites tumor cells washed in the same way. The mixture was incubated at 25° C for 30 min and then at 0° C for 60 min. The cells were removed by centrifugation at 1000 rpm for 5 min. In the case of the adsorption with lymphocytes, the procedure was done twice. The resulting supernatant was used as the adsorbed RS.

Assay of cytotoxicity. Splenocytes of C3H/He mice, obtained 3 days after intraperitoneal transplantation of MM2 cells, were used as the effector cells of the RS-dependent in vitro cytotoxicity reaction [23]. The effector cells (1×10^6) were mixed with 1×10^4 ^{51}Cr-labelled target cells in 0.5 ml RPMI-1640 medium supplemented with 7% FCS. In adsorption studies, the gammaglobulin-depleted RS before or after the adsorption was added at a final concentration of 500× dilution of the original RS. MM2 and various lymphoblasts were used as the target cells. The mixture was incubated for 18 h in the case of tumor cells and 7 h in the case of lymphoblasts at 37° C in a CO_2 incubator. The cytotoxicity was expressed in terms of percentage specific lysis [23]. All determinations were carried out in duplicate. In the adsorption studies, results of repeated or different experiments were normalized by expressing them in terms of percentage decrease of the RS activity. Complement-dependent cytotoxic activity was assayed by the dye-exclusion method with mesenteric lymph node cells as the target cells [2].

Serum protein fractionation. A serum protein subfraction, precipitated between 0.35 and 0.50 saturation of ammonium sulfate at 4° C, was fractionated by starch-gel zone electrophoresis using a starch block of $10 \times 40 \times 1.5$ cm buffered with 0.07 M barbital buffer (pH 8.6). The hydrolyzed starch for gel electrophoresis was purchased from Connaught Laboratories, Willowdale, Ontario. After electrophoresis for 24 h at 4° C at 35 mA, the gel was cut into 1-cm-wide blocks and each gel portion was eluted with 10 ml water. The resulting β-globulin fraction was dialyzed against 0.01 M TRIS/HCl buffer (pH 8.6), applied on a 1-ml Mono-Q column of fast protein liquid chromatography (FPLC) apparatus (Pharmacia, Uppsala) and was eluted with a 0–0.5 M linear gradient of NaCl in the same buffer [25]. After rechromatography, an active fraction with symmetrical elution profile was obtained.

Radioiodination of the protein and polyacrylamide gel electrophoresis. The FPLC-purified material (10 μg) was labelled with ^{125}I by the chloramine T method [5] using 1 mCi carrier-free sodium [^{125}I]-iodide (NEN). The labelled material was separated from the free iodide by passage through a column of Sephadex G-50. Polyacrylamide gel elec-

Table 2. Adsorption of regressor serum (RS) activity with various tumor cells

Tumor cells used for adsorption	Origin of tumor cells	Decrease of RS activity (%) with MM2 target cells[a]
MM2	C3H/He	93.3± 4.1
MM46	C3H/He	73.9± 7.0
MM48	C3H/He	2.9±17.8
X5563	C3H/He	14.0±13.4
Meth A	BALB/c	66.2± 7.3
EL-4	C57BL/6	66.9±13.4

[a] Means ± SE of the results of two or three experiments. Percentage specific lysis of MM2 in the presence of unadsorbed material was 31.4±6.8%

Table 3. Adsorption of RS activity with lymphocytes from mice of various strains

Decrease of RS activity[a] (%)	Origin of lymphocytes used for adsorption[b]
0– 5	C3H/He (k), CBA (k)
5– 20	None
21– 40	CE (k), AKR (k)
41– 60	C58 (k), BALB/c (d), DBA/1 (q), AJ (a)
61– 80	C57BL/6 (b), DBA/2 (d)
81–100	None

[a] Mean values of the results of two experiments in which MM2 cells were used as the cytotoxicity target cells

[b] H-2 haplotypes are shown in the parentheses

trophoresis (PAGE) of the labelled material was carried out using 7.5% or 10% gels containing 0.1% sodium dodecyl sulfate (SDS) [3]. Two-dimensional PAGE was carried out using nonequilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE after reduction of the material in the second dimension [13].

Detection of Qa-2 antigen by flow cytometry. A T-cell-enriched fraction was obtained from mouse splenocytes by adsorption to nylon wool [7]. Mouse ascites tumor cells were harvested 4–6 days after intraperitoneal transplantation. BW5147 cells were harvested at subconfluent stage. The cells were washed two times with MEM. Cell-surface immunoglobulins of the contaminating B cells were blocked by treating with the Fab′ fraction prepared from a goat anti-(mouse immunoglobulin) antibody for 60 min at room temperature. The cells were then reacted with Qa-2-specific mAb (141-15.8, 200× dilution). As controls, normal B6.K1 mouse serum or the mAb against a bacterial component were used. The cells were incubated with the mAb for 90 min and then with the FITC-labelled $F(ab')_2$ fraction of goat antibody specific to mouse immunoglobulin for 60 min at room temperature. All reagents were diluted in MEM containing 10% goat serum and 1% bovine serum albumin (BSA). Incubations were carried out in 50-μl volumes and the cells were washed two times after each incubation with phosphate-buffered saline (PBS) using centrifugation at 5000 rpm for 2 min in a microcentrifuge. The FITC-labelled cells were fixed with 1% paraformaldehyde and stored at 4° C until they were analyzed using a FACSIII apparatus (Becton Dickinson, Mountain View, Calif.).

Detection of serum IgD by enzyme-linked immunosorbent assay (ELISA). For the detection of Qa-2-specific IgD, a fusion protein constructed from extracellular domains of Qa-2 antigen and β-galactosidase was used. Production of the fusion protein in *E. coli* by isopropylthiogalactoside induction is described elsewhere [15]. The cells after the induction were harvested, lysed with 50% acetic acid, centrifuged to remove insoluble materials, made 1% with respect to BSA, dialysed extensively against PBS and used for ELISA. Flat-bottomed 96-well plates (Immunoplate, Nunc) were coated either with the *E. coli* extract or with sheep anti-(mouse IgD) serum both diluted with 50 mM carbonate buffer (pH 9.0) and then re-coated with 3% BSA. To each of the precoated and washed wells, 50 μl sample serum, serially diluted in PBS, was added and kept for 2 h at room temperature. The solution was discarded and the wells were washed three times with PBS. Then 50 μl solution of peroxidase-labelled rat mAb specific to mouse IgD, diluted with 1% BSA, was added. After standing at room temperature for 1 h, the solution was discarded and the wells were washed four times with PBS. A 100-μl sample of a mixture of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) solution and hydrogen peroxide solution (Kirkegaard and Perry Laboratories, MD) was then added to each well and allowed to stand for 5 min at room temperature; the reaction was terminated by adding 100 μl 1% SDS solution. The absorbance at 410 nm of the solution was determined.

Results

Recognition specificity of RS factors

It has been reported that splenocytes, obtained from C3H/He mice bearing ascites tumor cells 3 days after inoculation, lyse certain syngeneic (MM2, MM46 etc) and allogeneic (Meth A, EL-4 etc.) tumor cells by an RS-dependent cellular cytotoxic reaction. Some syngeneic tumor cells (MM48, X5563 etc.) were lysed less strongly by the reaction [23]. In this study we found that the activity of RS decreased after preincubation with tumor cells that were susceptible to the reaction. The decrease was lower when the tumor cells that were resistant to the reaction were used (Table 2). These results suggest that the active components in RS (referred as RS factors) had adsorbed to the target cell surface. Susceptibility of the allogeneic tumor cells in general prompted us to consider the possibility that RS factors recognize allogeneic antigens that might be expressed illegitimately on the tumor cell surface. To test this possibility, RS was adsorbed by lymphocytes obtained from mice of various strains and assayed using MM2 target cells. As shown in Table 3, the active components of RS were adsorbed by lymphocytes with H-2 haplotypes of k (CE, AKR and C58) and those with H-2 haplotypes other than k (B6, BALB/c, DBA/2, DBA/1 and A/J), but not by lymphocytes from C3H/He and CBA mice (both $H\text{-}2^k$). After correlating the results of the adsorption studies with the various allotypes of lymphocyte antigens, we speculated that the RS activity was related to allotypes of lymphocyte marker antigens coded by genes in the Qa/Tla region and also to allotypes of Ly groups. The results shown in Table 3 confirm this idea since adsorption of the RS factors occurred when allotypes of one or some of the antigen in the Ly group of lymphocytes differed from those of C3H/He, or when one or some of the Qa/TL antigens were expressed on the lymphocytes. In order to test this hypothesis further, double-absorption experiments were performed (Table 4). Partial decrease of the RS activity was observed after incubation with C58 lymphocytes. The decrease of the activity after this absorption suggests that RS contains factor(s) that bind to one or some of the Ly1.2, Ly3.1, Ly6.2, Ly7.1, Qa-1 and TL antigens, on the assumption that antigens of the Qa/TL or Ly groups are binding targets for RS factors. Factors that bind to either Ly2.2, Ly4.2, Ly5.2, Qa-2 or Qa-3, if the serum had any of them,

Table 4. Double absorption of RS activity with lymphocytes from mice of various strains

Origin of lymphocytes used		Decrease of RS activity (%) with MM2 target cells[a]
1st absorption	2nd absorption	
C58	C3H/He	37.8±11.5
	C57BL/6	97.4± 2.6
	B6.K1	33.1± 2.2
	B6.K2	94.1±14.4
	DBA/1	99.6± 6.3
	BALB/c	50.7± 6.8
C57BL/6	C3H/He	57.5± 4.3
	A/J	95.7± 1.1
A/J	C3H/He	38.4± 5.6
	C57BL/6	92.4± 0.8
	DBA/2	96.0± 2.8
	DBA/1	42.8± 0.4
	BALB/c	55.2± 0.4

[a] Means ± SE of the results of two experiments. RS preadsorbed doubly with C3H/He lymphocytes was used in the control experiments, and the values were 27.0±3.0% for C58 (1st absorption), 25.4±1.0% for C57BL/6 (1st absorption), and 25.0±1.3% for A/J (1st absorption)

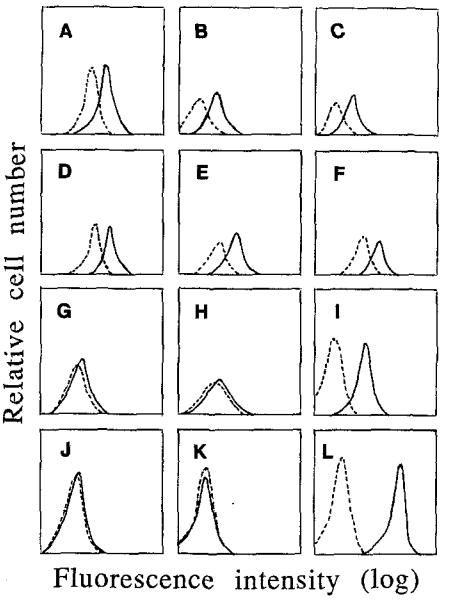

Fig. 1 A–L. Detection of Qa-2 antigen on various tumor cells derived from H-2^k mice and lymphocytes. Qa-2-specific mAb 141-15.8 (———) was used, and a mAb specific to a bacterial component (– – – –) was used as a negative control. Tumor cells used were MH134 (**A**), MH125F (**B**), MH125P (**C**), MM2 (**D**), FM3A (**E**), MM102D (**F**), MM46 (**G**), MM48 (**H**) and BW5147 (**I**). A nylon-wool-nonadherent fraction of splenocytes from C3H/He (**J**), B6.K1 (K) and B6.K2 (**L**) mice was also examined

would remain unadsorbed. The remaining activity decreased almost completely on incubation with B6, B6.K2 or DBA/1 lymphocytes. Neither B6.K1 nor BALB/c lymphocytes adsorbed the active factor in the preadsorbed RS significantly. These results suggest that the Qa-2 antigen adsorbs some of the factors. Likewise, possible contributions to the reaction by the antigens of the Qa-1/TL group were also investigated by adsorption of RS with B6 lymphocytes and then with A/J lymphocytes. The result indicated that one or some of the antigens that belong to the Qa-1/TL group may adsorb some portion of the RS factors. The activity of RS preadsorbed with A/J splenocytes and assayed using MM2 targets decreased almost completely after incubation with DBA/2 or B6 lymphocytes, but not after incubation with DBA/1 or BALB/c lymphocytes. This suggests that the Ly6.2 antigen adsorbs a part of the RS activity. Taken altogether, these results show that MM2 cells express Qa-2, Ly6.2 and some antigens in the Qa-1/TL group, and that RS contains multiple factors that bind specifically to each one of them. Phenotypes of all of these antigens are allogeneic to C3H/He mice. The results of the double-adsorption studies shown above also show that RS does not contain detectable amount of factors that recognize Qa-3, Ly2.2, Ly3.1, Ly4.2, Ly5.2 and Ly7.1.

In agreement with the above observations, various allogeneic lymphoblasts that express one or some of these antigens (CE, C58, C57BL/6, B6.K1, B6.K2, DBA/1, DBA/2, A/J and C3H-Ly6.2), but not C3H/He and AKR lymphoblasts, were lysed by the RS-dependent cell-mediated reaction (Table 5). The lysis of C3H-Ly6.2 lymphoblasts but not C3H/He lymphoblasts directly shows the contribution of the Ly6.2 antigen. The presence of a Qa-2-specific factor was confirmed by adsorption of the RS activity by lymphocytes of Qa-2,3-congenic mice (Table 6). The activity of RS to support lysis of B6 and B6.K2 lymphoblasts was adsorbed only partly by B6.K1 but completely by B6 or B6.K2 lymphocytes.

Demonstration of the Qa-2 antigen by flow cytometry

The presence of the Qa-2 antigen on the surface of MM2 and several other tumor cell lines derived from H-2^k (Qa-2-) mice was demonstrated more directly by the membrane immunofluorescence method using Qa-2-specific mAb 141-15.8. As shown in Fig. 1, significant Qa-2-specific fluorescence was detected reproducibly in seven of the nine cell lines tested. These results show that expression of the Qa-2 allo-antigen on tumor cells is not an event specific to MM2 cells but is common among various lymphoma and nonlymphoma cell lines and seems to account partly for the wide target selectivity of the RS-dependent reaction.

Characteristics of the Qa-2-specific RS factor

Among multiple factors recognizing allo-antigens presumed to be present in RS, the specificity of a factor to Qa-2 was most clearly demonstrated as described above. We therefore focussed our attention on this factor for further characterization. By ammonium sulfate precipitation and preparative electrophoresis of the serum protein, the activity to support the cell-mediated lysis of B6 lymphoblasts, but not of B6.K1 lymphoblasts, was found mainly in the β-globulin fraction. This fraction was then fractionated by FPLC using the Mono-Q column. The active component was eluted at 0.30 M NaCl, whereas al-

Table 5. RS-dependent cell-mediated lysis of various lymphoblasts

Origin of the target lymphoblast[a]	Specific lysis[b] (%)	
	Without RS	With RS[c]
C3H/He	0.3±1.2	4.2± 5.2
C3H-Ly6.2	0.0±0.6	30.5±12.5
CE	0.4±0.3	20.1± 3.5
C58	0.2±0.4	31.6± 5.1
C57BL/6	5.2±4.8	37.5±12.0
B6.K1	0.1±0.2	33.2± 4.9
B6.K2	0.1±0.3	37.1± 7.0
DBA/1	4.8±5.3	32.8± 2.7
DBA/2	2.3±3.2	31.4± 1.5
A/J	0.3±0.2	36.7± 1.2

[a] Lymphoblasts were prepared by culturing the lymphocytes in the presence of 5 μg/ml concanavalin A (Sigman, St. Louis) for 3 days
[b] Means ± SE of the results of two experiments
[c] Gammaglobulin-depleted RS was added at a final concentration of 500× dilution

Table 6. Demonstration of Qa-2 specificity of RS activity by adsorption studies

Lymphocytes used for adsorption	Decrease of RS activity[a] (%) with target lymphoblasts	
	B6.K1	B6.K2
C3H/He	0.0±1.0	−1.9±11.9
B6.K1	95.8±8.1	11.6±17.8
B6.K2	95.1±3.9	96.9± 1.4

[a] Means ± SE of the results of two experiments. Gammaglobulin-depleted RS was adsorbed with lymphocytes and added to the reaction mixture at a final concentration of 500× dilution. Values of percentage specific lysis of B6.K1 and B6.K2 lymphoblasts in the presence of unadsorbed RS were 28.3±1.2% and 35.4±2.2% respectively

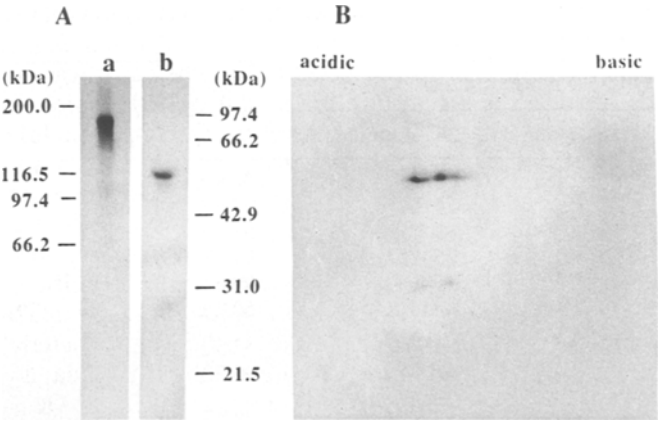

Fig. 2 A, B. Autoradiograms of the ^{125}I-labelled regressor serum (RS) component adsorbed to cells and analyzed by polyacrylamide gel electrophoresis (PAGE). Approximately 100 ng radiolabelled fast-protein-liquid-chromatography-purified material was dissolved in 0.2 ml Eagle's minimum essential medium (MEM) containing 7% fetal calf serum. The solution was mixed with 2×10^7 C58 mesenteric lymph node cells and kept at room temperature for 60 min and then on ice for 30 min. The cells were then washed twice with MEM and dissolved in 50 μl 50 mM TRIS buffer (pH 6.8) containing 4% sodium dodecyl sulfate (SDS), 10% glycerol, with or without addition of 10% 2-mercaptoethanol. The mixture was heated at 100° C for 5 min and centrifuged at 5000 rpm for 2 min. The supernatant wa analyzed by gel electrophoresis. **A** One-dimensional SDS-PAGE under non-reducing (*a*) and reducing (*b*) conditions; a 7.5% polyacrylamide gel was used. Positions of migration of standard substances with known molecular mass (kDa) are shown. **B** Pattern of two-dimensional gel electrophoresis. The material under non-reducing conditions was separated by nonequilibrium pH gradient gel electrophoresis for the first dimension, reduced and then electrophoresed through a 10% gel containing 0.1% SDS for the second dimension

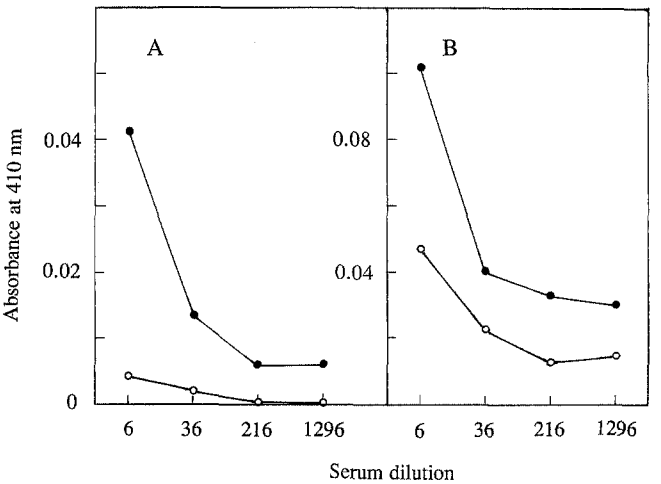

Fig. 3 A, B. Enzyme-linked immunosorbent assay for IgD in RS and normal C3H/He serum. Microtiter plates coated with Qa-2–lacZ fusion protein were used for the detection of Qa-2-specific IgD (**A**) and those coated with sheep antiserum monospecific to mouse IgD were used for the detection of total serum IgD (**B**)

most all of the mouse mAbs of the IgG subclasses and those of the IgM class were eluted at around 0.25 M and 0.35 M NaCl positions respectively. The semi-purified material was also tested for complement-dependent cytotoxic activity against Qa-2$^+$ lymphocytes. Complement-dependent lysis of B6 mesenteric lymph node cells by the FPLC-purified material was not detected even when 35 μg protein was used per assay. On the other hand, Qa-2-specific mAb of the IgG2b class lysed the same target cells in the presence of complement at an antibody concentration as low as less than 0.5 μg protein/assay. To characterize further the Qa-2-specific RS component, the FPLC-purified material was radiolabelled with ^{125}I, preadsorbed with C58 lymphocytes, adsorbed to the surface of Qa-2$^+$ cells and analyzed by SDS-PAGE or by two-dimensional PAGE followed by autoradiography (Fig. 2). About 2% of the total radioactivity was observed to bind specifically to the cells. The relative mass of the bound material under non-reducing conditions was estimated to be 160 kDa. Under reducing conditions, it showed bands with relative masses of approximately 50 kDa and 25 kDa. These results revealed that the material had a heavy and light chain structure similar to that of IgG. The two-dimensional gel electrophoresis of the labelled material showed that it was composed of four or more components with isoelectric points ranging approximately from 5.5 to 6.5 and all having heavy and light chain structures. On the basis of these characteristics we speculated that the Qa-2-specific factor was an IgD (see Discussion), and this possibility was tested using an

ELISA with Qa-2-specific mAb and microtiter plates coated with Qa-2–lacZ fusion protein. As shown in Fig. 3, Qa-2-specific IgD was detected in RS whereas it was not detected in the serum of normal adult C3H/He mice. In accordance with the appearance of the Qa-2-specific IgD, an increase of the total amount of serum IgD was also detected in RS compared to the normal control serum. Minor amounts of Qa-2-specific IgG2a and IgG2b were also detected in RS by ELISA using the fusion-protein-coated plates and peroxide-labelled rat mAbs specific to mouse IgG2a and IgG2b respectively. Qa-2-specific IgG2a and IgG2b were not detected in the FPLC-purified material while Qa-2-specific IgD was detected in it (data not shown). From these results, it is very likely that the Qa-2-specific activity in RS is mediated by an IgD.

Discussion

Results of the present study indicate that MM2 cells express antigens on their surfaces to which host C3H/He mice responded by producing antibodies that are cross-reactive to allogeneic lymphocyte antigens including Qa-2, Ly6.2 and some antigens in the Qa-1/TL group. These conclusions were drawn from all of the results obtained using splenocytes from the 13 different mouse strains used. Similar experiments showed that some of the Qa-1, Qa-2 and TL antigens may also be expressed on Meth A cells derived from a BALB/c hepatoma (data not shown). In the Qa/Tla gene region, which is adjacent to the H-2 region of mouse chromosome 17, numerous non-classical histocompatibility class 1 antigen genes are tandemly arranged. They are highly conservative whereas H-2 genes are polymorphic. The physiological roles of the non-classical histocompatibility class 1 antigens are not yet known. However, it has been shown that serologically defined Qa-2 antigens detected on lymphocytes are coded for by the $Q7^b$ and $Q9^b$ genes of the Qa-2,3 subregion of B10 mice ($H\text{-}2^b$) and by the $Q7^d$ gene of the $Qa\text{-}2^+$ subline of BALB/c mice ($H\text{-}2^d$) [11, 18, 27]. In contrast, Watts et al. (1989) reported that $H\text{-}2^k$ mice lack the genes for Q7 and Q9. Therefore the Qa-2 molecule expressed on tumor cells derived from $H\text{-}2^k$ mice should be the product of a gene or genes other than Q7 and Q9. We reported elsewhere that the serologically defined Qa-2 molecule expressed on $H\text{-}2^k$ tumor cells ($Qa\text{-}2^k$ antigen) is encoded by the $Q5^k$ gene, and is distinct from the $Q7^b$ gene product in some respects [15]. The results shown here, however, do not concure with this since the Qa-2-specific factor in RS, which was assayed using the tumor cells expressing the $Qa\text{-}2^k$ antigen, adsorbed to lymphocytes expressing the Q7 or Q9 gene product. This result shows that the RS factor produced as a host response to $Qa\text{-}2^k$ tumor antigen cross-reacts with the normal Qa-2 allo-antigen. Many human as well as mouse tumor cells have been reported to express cell-surface antigens cross-reactive with those expressed on normal cells of unrelated individuals of the same species [14]. Also, cytotoxic T lymphocytes to human leukemia cells are generated by in vitro sensitization of the peripheral blood T cells of the patients with allogeneic normal lymphocytes [28]. These reports indicate that tumor antigens that have potential immunogenicity in the hosts include those cross-reactive to allogeneic lymphocyte antigens. The results presented here reveal the possibility that at least some such tumor antigens are non-classical histocompatibility class 1 antigens. Involvement of non-classical histocompatibility class 1 antigens in the immunological responses by patients with malignancies will be a subject of investigation for the development of a novel means of cancer immunotherapy.

Serum IgD is a minor component of immunoglobulins. IgD are found in the β-globulin fraction of serum proteins and comprise acidic glycoproteins [6], whereas IgGs in general are basic proteins. IgD has a structure composed of heavy and light chains like IgGs, but does not interact with C1q of the complement system [19, 20]. It has also been reported that there are two forms of mouse serum IgD. One has a molecular mass of about 170–200 kDa, while the other, which lacks the C1 domain of the heavy chains, has a molecular mass of approximately 150–160 kDa [4, 12]. Variety in the sugar moieties of a human monoclonal IgD (NIG-65 myeloma protein) seems to cause heterogecity in the isoelectric point between 5.6 and 6.8 [17]. All of these characteristics agree with those determined for the serum component having Qa-2 specificity reported here. Qa-2-specific IgD was also demonstrated by ELISA. These results indicated that the Qa-2-specific component in RS is an IgD. The possibility remains that the RS factor actually responsible for the cytotoxic reaction is an IgG, but this possibility is not likely since Qa-2-specific IgGs are undetectable in the FPLC-purified material and depletion of IgGs does not affect the activity. The role of B cell surface IgD as an antigen-recognition molecule is well known; however, the physiological roles of serum IgD are unknown. The results presented in this report indicate that target cells are lysed by an IgD-dependent cell-mediated reaction. To elucidate such an activity of IgD, further studies will be necessary including the generation of Qa-2-specific mAb of the IgD class, establishment of the IgD-dependent cytotoxic cell clone, characterization of the IgD receptor on the effector cells, etc. These studies are under trial in our laboratory.

Acknowledgements. We are grateful to Dr. K. Sudo of the Laboratory Animal Research Center of this institute for collection of mice of various syngeneic strains, Dr. T. Takahashi of Aichi Cancer Center Research Institute for generously providing B6.K1, B6.K2 and C3H-Ly6.2 mouse strains, Dr. H. Nagayama of Tohoku University for providing various tumor cell lines, Miss T. Sugimoto for assistance during preparation of the manuscript, and Dr. B. Powell for critical reading of the manuscript. This work was supported in part by a grant from the Ryoichi Naito Foundation for Medical Research.

References

1. Boyle W (1968) An extension of the ^{51}Cr-release assay for the estimation of cytotoxins. Transplantation 6: 761
2. Boyse EA, Old LJ, Chouroulinkov I (1964) Cytotoxicity test for demonstration of mouse antibody. Methods Med Res 10: 39
3. Davis BJ (1964) Disc electrophoresis: II. Method and application to human serum proteins. Ann NY Acad Sci 121: 404
4. Finkelman FD, Kessler SW, Mushinski JF, Potter M (1981) IgD-secreting murine plasmacytomas: identification and partial characterization of two IgD myeloma proteins. J Immunol 126: 680

5. Greenwood HC, Hunter WM, Glover JS (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 89: 114
6. Ishihara E, Tejima Y, Takahashi R, Takayasu T, Shinoda T (1983) Structure and location of asparagine-linked olygosaccharides in the Fc region of a human immunoglobulin D. Biochem Biophys Res Commun 110: 181
7. Julius MH, Simpson E, Herzenberg LA (1973) A rapid method for isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 3: 645
8. Kimura Y, Tanino-Komaiji T (1966) Survival of host mice and induced resistance to transplantable ascites tumors. Jpn J Exp Med 36: 371
9. Klein J, Figueroa F, David CS (1983) H-2 haplotypes, gene and antigens: second listing. Immunogenetics 17: 553
10. McKenzie IFC, Potter T (1979) Murine lymphocyte surface antigens. Adv Immunol 27: 179
11. Mellor AL, Antoniou J, Robinson PJ (1985) Structure and expression of genes encoding Qa-2 class I gene. Proc Natl Acad Sci USA 82: 5920
12. Mountz JD, Mushinski JF, Owens JD, Finkelman FD (1990) The in vivo generation of murine IgD-secreting cells is accompanied by deletion of Cμ gene and occasional deletion of the gene for the Cδ1 domain. J Immunol 145: 1583
13. O'Farrell PZ, Goodman HM, O'Farrell PH (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133
14. Rigby PW, Brickell PM, Latchman DS, Murphy D, Scott MRD, Westphal KH, Simains V, Lane DP, Willison K (1984) Oncofetal genes and major histocompatibility complex. In: Cancer cells: transformed phenotype. Cold Spring Harbor Laboratory, New York, p 97
15. Seo N, Okazaki T, Nakanishi-Ito C, Tanino T, Matsudaira Y, Takahashi T, Egawa K (1992) Expression of the Qa-2^k phenotype encoded by the Q5^k gene on the surface of tumor cells derived from H-2^k mice. J Exp Med 175: 647
16. Sharrow SO, Arn JS, Stroynowski I, Hood L, Sachs DH (1989) Epitope clusters of Qa-2 antigens defined by a panel of new monoclonal antibodies. J Immunol 142: 3495
17. Shinoda T (1983) Primary structures of immunoglobulins. In: Onoe K, Utsumi S (eds) Immunoglobulins. Iwanami Shoten, Tokyo, p 15
18. Soloski MJ, Hood L, Stroynovski I (1988) Qa-region class I gene expression: Identification of a second class I gene, Q9, encoding a Qa-2 polypeptide. Proc Natl Acad Sci USA 85: 3100
19. Spiegelberg HL (1977) The structure and biology of human IgD. Immunol Rev 37: 3
20. Spiegelberg HL (1985) Immunoglobulin D (IgD). Methods Enzymol 116: 95
21. Stanton TH, Boyse EA (1976) A new serologically defined locus, Qa-1, in the Tla-region of the mouse. Immunogenetics 3: 525
22. Tanino T (1968) Studies on tumor-host relationship: II. Serum resistance factor(s) produced by tumor transplantation. Jpn J Exp Med 38: 383
23. Tanino T, Egawa K (1986) Regressor serum factor-dependent nonspecific killers in tumor-bearing mice. Jpn J Exp Med 56: 163
24. Tanino T, Saito M, Egawa K (1976) Cell line-specific cell surface component of hypotetraploid ascites mammary carcinoma cells inducing humoral reactions in the syngeneic host. Int J Cancer 17: 736
25. Tomono T, Ikeda H, Tokunaga E (1983) High-performance ion-exchange chromatography of plasma proteins. J Chromatorgr 266: 39
26. Watts S, Davis AC, Gaut B, Wheeler C, Hill L, Goodenow RS (1989) Organization and structure of the Qa genes of the major histocompatibility complex of the C3H mouse: implications for Qa function and class I evolution. EMBO J 8: 1749
27. Weiss EH, Golden L, Fahrner K, Mellor AL, Devlin JJ, Bullman H, Tiddens H, Bud H, Flavell RA (1984) Organization and evolution of the class I gene family in the major histocompatibility complex of the C57BL/10 mouse. Nature 310: 650
28. Zarling JM, Robins HI, Raich PC, Bach FH, Bach ML (1978) Generation of cytotoxic T lymphocytes to autologous human leukemia cells by sensitisation to pooled allogeneic normal cells. Nature 274: 269