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Identification and Characterization of a Unique Tumor-associated Surface Antigen on L1210 Leukemia Cells Recognized by Semisyngeneic Antisera[1]

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ABSTRACT

The tumor-associated surface antigen on L1210 leukemia cells was studied by immunofluorescence staining and immunoprecipitation. Anti-L1210 serum was prepared in BALB/c × DBA/2 F_1 mice by priming with a hybrid of L1210 and human Lesch-Nyhan fibroblast cells and hyperimmunizing with L1210 leukemia cells. This hyperimmune serum was able to demonstrate specific surface fluorescence on L1210 cells, while the antiserum did not react with various mouse tumor cell lines, normal lymphoid tissues, or mitogen-activated lymphoid cells. The anti-L1210 serum immunoprecipitated a single polypeptide with a molecular weight of 90,000 from ^{125}I-labeled L1210 cells. The expression of this antigen was enhanced by tumor-promoting agent and heat shock treatment. The biological significance of the L1210-specific cell surface antigen is discussed.

INTRODUCTION

Many attempts have been made to raise antisera to serologically defined TASAs[3] in order to characterize these antigens in relation to TATAs. Nevertheless, there are very few reports on antibodies showing specificity for the immunizing syngeneic tumor (1). Recently Kawashima *et al.* (2) have succeeded in inducing highly effective immunity against transplantation of L1210 murine leukemia cells by initially immunizing BALB/c × DBA/2 F_1 mice with a L1210 variant (L1210/LN-1) which was derived from the long-term culture of a hybrid of L1210 and human Lesch-Nyhan fibroblasts. This high-grade tumor-specific immunity was due to cell-mediated immunity (2). However, humoral antibody against L1210 cells also developed in hyperimmunized mice (3). In the present study we investigated the serological specificity of the anti-L1210 serum from hyperimmunized mice and the biochemical property of the antigen detected with this antiserum.

MATERIALS AND METHODS

Mice. Inbred BALB/c × DBA/2 F_1 (hereafter called CD2F$_1$) mice was obtained from Shizuoka Experimental Animal Cooperative, Hamamatsu, Japan. Six- to 8-week-old females were used in this study.

Cell Lines. L1210, P388, L5178Y, P815, WEHI 3, WEHI 279.1, FDC-P2, Yac-1, BW5147, MMTV-73, SP-2, B16, P3U1, L, and LS-1 cell lines were used in this study, and their origins and properties are listed in Table 1. These cell lines except MMTV-73 were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, antibiotics, and 10% fetal calf serum. MMTV-73 was maintained in this culture medium containing 2 μg dexamethasone and 10 μg insulin.

Production of Anti-L1210 Serum. The immunization schedule was described previously (2). Briefly, CD2F$_2$ mice were primed i.p. with 2 × 10^6 L1210/LN-1 cells and then followed up with seven i.p. booster challenges with viable 1 × 10^7 L1210 cells at intervals of 4 weeks. Antisera were collected 4 weeks after the last inoculation.

Normal Tissues. Mouse spleen, mesenteric lymph nodes, and thymus were dissected to prepare a single-cell suspension in RPMI 1640 containing 10% fetal calf serum. Bone marrow cells were collected by flushing out two pieces of femur shafts.

Activation of Lymphocytes. Spleen cells and thymocytes (2 × 10^6/ml) were cultured in the medium described above containing 5 × 10^{-5} M 2-mercaptoethanol in 24-well plates with 5 μg/ml Con A (Sigma Chemical Co., St. Louis, MO) or 0.01 ml/ml phytohemagglutinin (Difco Laboratories, Detroit MI). Two days later cells were washed and used for the immunofluorescence assay.

Flow Cytometry Analysis. Cells were treated with a 1:100 dilution of anti-L1210 serum or normal mouse serum for 30 min at 4°C and then stained with fluorescein-conjugated goat F(ab′) anti-mouse IgG serum (1:40) (Tago, Inc., Burlingham, CA). Immunofluorescence-positive cells were analyzed with the aid of a cytofluorograph (Cell sorter CS-20; Showa Denko K. K., Japan). The intensity of fluorescence is expressed in log scale.

Labeling of Protein, Immunoprecipitation, and Gel Electrophoresis. Cell surface proteins of L1210 cells were labeled by the ^{125}I-lactoperoxidase method (4). Cell lysates were prepared using 0.5% Nonidet P-40 detergent in 0.15 M NaCl, 0.05 M Tris, and 5 mM EDTA at pH 8.0 (5) and the lysate was adjusted to a concentration of 0.1% SDS. The lysate was immunoprecipitated with antibody and protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Radiolabeled proteins were analyzed by polyacrylamide gel electrophoresis in the presence of SDS in 10% slab gel under reducing or nonreducing conditions (6). Radiolabeled bands in the gels were identified by autoradiography. Molecular weights were determined by calculating the correlation of the log of the molecular weight with migration distance. A pharmacia low molecular protein standard kit was used as reference.

Heat Shock Conditions. L1210 leukemia cells were heat shocked by placing a sealed culture dish in a 43°C water bath for 20 min. Control cultures were placed in a 37°C water bath for 20 min. The cells were then allowed to recover in a 37°C incubator. There was no significant decrease in the cell viability of the samples.

RESULTS

Reactivity of Anti-L1210 Serum against Various Kinds of Mouse Cell Lines. A screen against a panel of cell lines revealed that the antigenic determinant recognized by anti-L1210 serum was expressed exclusively on L1210 cells and only marginally on P388 cells (Table 1). A typical histogram of immunofluorescence in L1210 cells is shown (Fig. 1). However, P388 cells failed to absorb L1210-specific immunofluorescence reactivity from the antiserum, indicating that anti-L1210 serum reacted nonspecifically with P388 cells. All other cell lines containing SP-2 (myeloma cells), WEHI 3 (myelomonocytic cells), WEHI 279.1 (B-lymphoma cells), B16 (melanoma cells), L (fibroblast cells), P3U1 (myeloma cells), and FDC-P2 (long term-cultured marrow cells) were not reactive with the antiserum, and no absorption of the specific reactivity by these cell lines was confirmed.

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[3] The abbreviations used are: TASA, tumor-associated surface antigen; TATA, tumor-associated transplantation antigen; Con A, concanavalin A; SDS, sodium dodecyl sulfate; TPA, tumor promoting agent (12-*O*-tetradecanoylphorbol-13-acetate).

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Reactivity of Anti-L1210 Serum against Normal Lymphoid Tissues. The histogram of spleen cells stained with anti-L1210 serum was analyzed by flow cytometry. As shown in Fig. 2, the pattern of immunofluorescence staining of spleen cells was identical to that of cells treated with normal mouse serum. Thymocytes, bone marrow cells, and mesenteric lymph node cells were also negative for immunofluorescence staining.

Reactivity of Anti-L1210 Serum against Mitogen-activated Lymphocytes. To investigate the possibility that the anti-L1210 serum may recognize a kind of cellular antigen appearing at the stage of blastogenesis or on dividing cells, the reactivity against Con A-activated or phytohemagglutinin-activated spleen cells and thymocytes was studied by the flow cytometry. However, no specific fluorescence could be detected on Con A-activated spleen cells and thymocytes (Fig. 3) and phytohemagglutinin-activated cells (data not shown).

Biochemical Characteristics of Cell Surface Antigen Recognized by Anti-L1210 Serum. Anti-L1210 serum immunoprecipitated a polypeptide with an apparent molecular weight of 90,000 when the SDS gel was run under either nonreducing or reducing conditions (Fig. 4). Anti-L1210 serum absorbed with L1210 cells did not immunoprecipitate the M_r 90,000 antigen, whereas antiserum absorbed with P388 cells still did it.

Comparison of Reactivity of Anti-L1210 Serum in L1210 Cells and L1210/LN-1 Cells. Since the administration of L1210/LN-1 variant cells, but not L1210 cells, triggered both anti-L1210 humoral and cell-mediated immune responses *in vivo* (2, 3), it was of interest to see how the L1210/LN-1 variant cells carried the antigen recognized by anti-L1210 serum. The cytofluorograph analysis showed that the antigen expressed on L1210/LN-1 variant cells was about twice as much as L1210 cells. (Fig. 5).

Augmented Reactivity of the Antiserum against TPA-treated L1210 Cells. L1210 cells were cultured in the medium containing 100 ng/ml TPA for 2 days. The reactivity of the antiserum against TPA-treated and untreated L1210 cells was compared (Fig. 6). The intensity of immunofluorescence on TPA-treated cells was more than twice as strong as that on untreated cells. The reactivity of L1210 cells treated with TPA for 48 h became greater than for 24 h. In addition, this enhancement was not seen on TPA-treated spleen cells, thymocytes, L5178Y, and P815 leukemia cells.

Table 1 *Reactivity of anti-L1210 serum against various kinds of mouse cell lines*

Cell line	Characteristics	Degree of positivity[a]
L1210	Methylcholanthrene-induced	6.4
P388	Methylcholanthrene-induced	2.0
L5178Y	Methylcholanthrene-induced	<1.5
LS-1	Methylcholanthrene-induced	<1.5
BW5147	Gross leukemia virus-induced	<1.5
Yac-1	Moloney leukemia virus-induced	<1.5
P815	Mastocytoma	<1.5
MMTV-73	Mouse mammary tumor line	<1.5

[a] Degree of positivity was quantitatively assessed by flow cytometry. The ratio of the peak fluorescence with anti-L1210 serum to that with normal mouse serum was calculated.

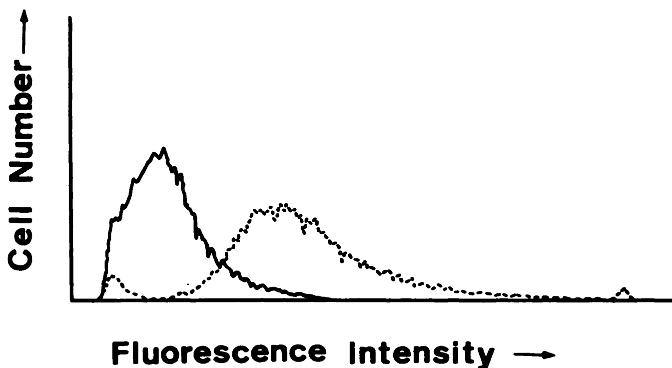

Fig. 1. Histogram of immunofluorescence on L1210 cells stained with anti-L1210 serum (——) or normal mouse serum (——).

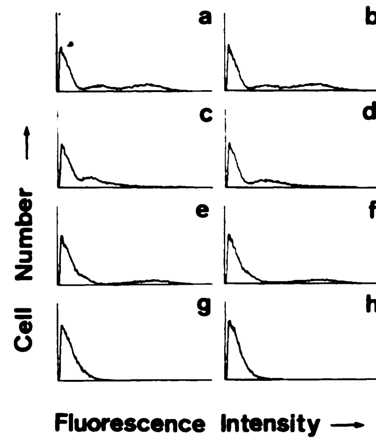

Fig. 2. Histogram of immunofluorescence on spleen cells (*a, b*), thymocytes (*c, d*), mesenteric lymph node cells (*e, f*), and bone marrow cells (*g, h*) stained with anti-L1210 serum (*a, c, e,* and *g*) or normal mouse serum (*b, d, f,* and *h*). No specific fluorescence was detected.

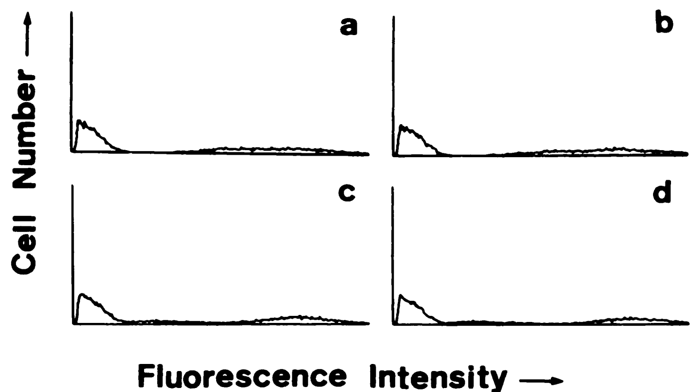

Fig. 3. Histogram of immunofluorescence on Con A-activated spleen cells (*a, b*) and thymocytes (*c, d*) stained with anti-L1210 serum (*a, c*) and normal mouse serum (*b, d*). No specific fluorescence was detected.

Enhanced Expression of the Antigen on Heat-shocked L1210 Cells. We examined whether the expression of the antigen on L1210 cells could be enhanced with heat shock treatment. In all four experiments, the expression of the antigen was markedly enhanced on L1210 cells 24 h after heat shock treatment (Fig. 7), whereas there was no increase in antigen expression 3 days after the treatment. At 6 h after treatment the immunofluorescence intensity was slightly increased in 2 of 4 experiments.

Effect of Anti-L1210 Serum on Cell Growth. We examined the effect of anti-L1210 serum on cell growth. The addition of antiserum to the cultures could not inhibit or stimulate the *in vitro* growth of L1210 cells and the colony formation in agar.

DISCUSSION

In the present study, we have defined a unique TASA on L1210 leukemia cells for the first time by the use of semisyngeneic antiserum obtained recently (2, 3). Several findings suggest that this antigen is fairly tumor specific: (*a*) the antigen is not found on either normal lymphoid cells or *in vitro*-activated cells; (*b*) the antigen is exclusively expressed on L1210 leukemia cells but not on any of the other 14 mouse cell lines tested. Our findings offer a remarkable contrast to the general

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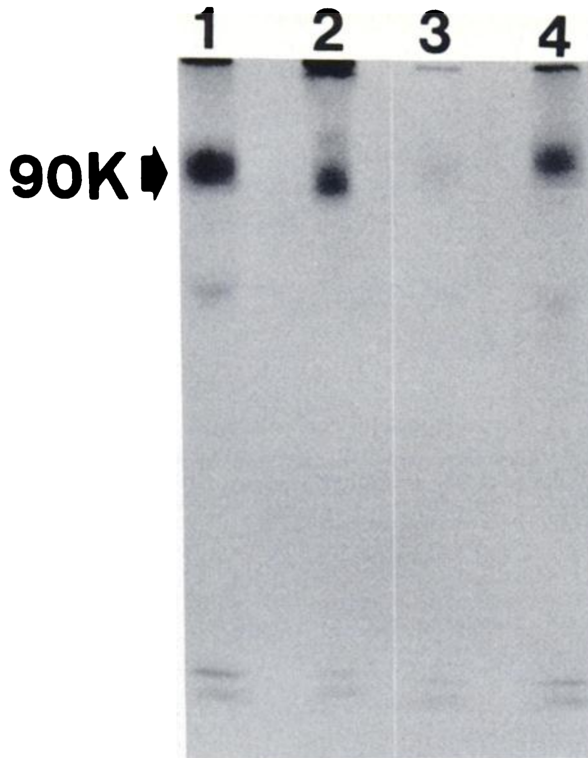

Fig. 4. SDS/polyacrylamide gel electrophoresis autoradiograph of ^{125}I-labeled protein precipitated from L1210 cells. Anti-L1210 serum precipitated M_r 90,000 (*90K*) polypeptide at reducing (*Lane 1*) and nonreducing (*Lane 2*) conditions. Anti-L1210 sera absorbed with L1210 cells (*Lane 3*) and P388 cells (*Lane 4*) were also used.

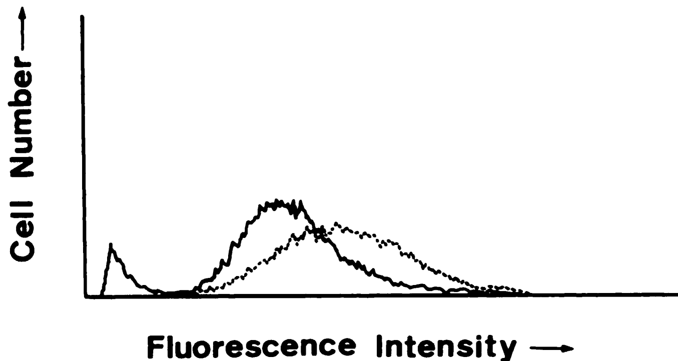

Fig. 5. Reactivity of anti-L1210 serum against L1210/LN-1 cells (——) and L1210 cells (——).

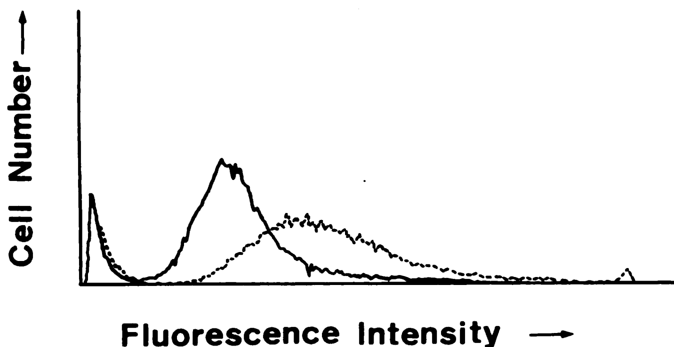

Fig. 6. Reactivity of anti-L1210 serum against TPA-treated L1210 cells (——) and untreated cells (——).

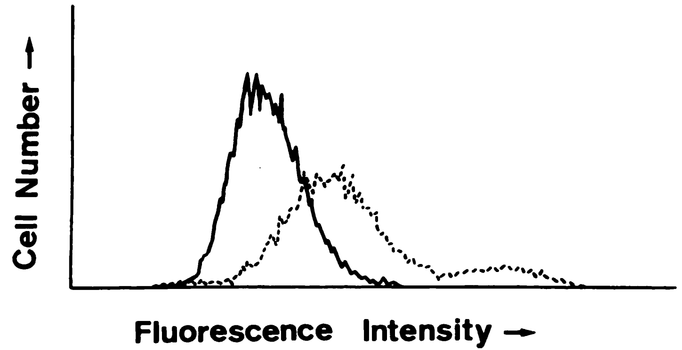

Fig. 7. Reactivity of anti-L1210 serum against heat-shocked L1210 cells (——) and untreated L1210 cells (——). L1210 cells were heat shocked at 43°C for 20 min and recovered at 37°C for 24 h.

conception that immunization with chemically induced syngeneic tumor cells barely induces antibody responses against the unique TATA, although endogenous mouse leukemia virus antigen does elicit high titers of antibodies (1). The M_r 90,000 antigen demonstrated on L1210 leukemia cells in the present study is not consistent with the known M_r 70,000 glycoprotein antigen of endogenous Gross and Rauscher murine leukemia viruses and M_r 73,500 antigen of mammary tumor virus which were previously shown to exist in L1210 cells (7, 8). In fact, the anti-L1210-specific antisera did not react with leukemia virus-induced tumor cell lines, such as Gross leukemia virus-induced BW5147 cells and Moloney leukemia virus-induced Yac-1 cells. Furthermore, rabbit antiserum against mammary tumor virus of various mouse strains did not immunoprecipitate the M_r 90,000 antigen from ^{125}I-labeled L1210 cells (data not shown), and the anti-L1210 antiserum was not reactive with mammary tumor virus-induced MMTV-73 cells. It is, therefore, unlikely that the L1210-specific M_r 90,000 antigen is related to the already known virus-associated antigens in L1210 leukemic cells. Our M_r 90,000 antigen is similar to tumor-associated antigens isolated from SV40-induced sarcoma (M_r 86,000) (9), Meth A and CMS 5 (M_r 96,000) (10), and methylcholanthrene-induced sarcoma CII-7 (M_r 82,000, 86,000) (11) but differs from the previously reported tumor-specific M_r 75,000 antigen on Meth A sarcoma (12, 13). Recently, Ullrich *et al.* (14) isolated the heat shock-related tumor specific transplantation antigen with its molecular weights of 84,000 and 86,000 from Meth A. This antigen is very close to our M_r 90,000 antigen enhanced by heat shock. The biochemical data also distinguish our antigen from class I and class II major histocompatibility antigens. The transferrin receptor, one of the cell surface antigens on activated cells, consists of a glycoprotein with an apparent molecular weight of 90,000, forming a M_r 200,000 disulfide-bonded dimer (15). However, our M_r 90,000 antigen exists as a single polypeptide chain and is not expressed on activated lymphocytes. Thus far, the biological function of the L1210-specific antigen is still a matter for speculation. However, it should be noted that the cell surface expression of the L1210-specific antigen was definitely augmented by TPA. Since phorbol esters induce the oncogene expression (16, 17) and proliferation (18–20) and differentiation (21–23) of various cell types, it is worth considering the possibility that the L1210 leukemia-specific antigen might be associated with oncogenicity and cell differentiation.

Previously Kawashima *et al.* (3) showed that the strong cell-mediated tumor immunity initiated by L1210/LN-1 cells is L1210 specific, and a cytotoxic T-cell clone with the same antigen specificity has been established (24). TASA detected

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serologically in the present study is also L1210 specific. Therefore, it is suggested that TATA for tumor rejection is very closely associated with TASA defined in this study. This idea is also supported by the fact that the L1210/LN-1 cell line, a highly immunogenic variant of L1210 cells for tumor rejection, showed augmented reactivity with the anti-L1210 serum. However, this antiserum totally failed to block lysis of L1210 cells by cytotoxic T-cells (3). This suggests that the antiserum and the cytotoxic T-cells may recognize different antigenic determinants, possibly on the same molecule. Further study is in progress to clarify the exact relationship between TASA and TATA on L1210 leukemia cells.

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