Characterization of a Monoclonal Antibody Reacting with a Neutral Glycolipid That Is Associated with Human T-Cell Leukemia Virus Type I Infection and Freshly Isolated Adult T-Cell Leukemia

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ABSTRACT

During attempts to make leukemia cross-reactive with human lymphocytes, we established a monoclonal antibody (VJ-41) from the alloimmunization of mice, that is, B10.A(3R) anti-B10.A(5R). VJ-41 reacted with all cases of freshly isolated adult T-cell leukemia cells (36 cases) but not with cells from other hematological disorders (more than 50 cases). Human T-cell leukemia virus Type I healthy carriers also seemed to possess these VJ-41 antigen positive cells. However, in vitro established adult T-cell leukemia cell lines did not show the reactivity with VJ-41. Normal lymphocytes from humans or mice apparently did not carry this antigen, but mitogen activated lymphocytes or some in vitro maintained cell lines of both human and mouse origin showed positive reaction. Having established solid phase radioimmunoassay to detect the VJ-41 antigen in plasma, it was found that healthy human T-cell leukemia virus Type I carriers, but not the majority of adult T-cell leukemia patients, predominantly possessed this antigen. Even though immunochemical characterizations of cellular materials were unsuccessful, a certain neutral glycolipid was detected from healthy human T-cell leukemia virus Type I carrier plasma by using thin-layer chromatography and immunostaining with the VJ-41 antibody.

INTRODUCTION

ATL has been recognized as a unique T-cell malignancy because of its restricted epidemiatic areas (1) and its relationship to the novel human T cell leukemia virus type-I (HTLV-I). This is the first example of a human malignancy, whose leukemogenesis has been found to be very closely related to the HTLV-I retrovirus infection (2—4). Indeed, all the leukemia cells from ATL patients carry the exogenous HTLV-I genome (5), and in the sera of these patients, antibodies against HTLV-A which are derived from the HTLV genome have been detected (6). However, freshly isolated leukemia cells from the ATL patients express neither the HTLV-A nor the transcription of the virus genome (7), throwing in doubt the concept of the HTLV-I virus playing a direct role in maintaining the leukemic state in vivo. For the study of this leukemia, many cell lines have been established by coculturing cord blood cells with ATL leukemic cells (8), or by culturing peripheral blood cells (PBC) from ATL patients in the presence of interleukin-2 (9). They have served as useful tools for such research, and these cell lines showed a high copy number of the HTLV-I in the genome. Recent studies of clonality analysis, however, revealed that these ATL cell lines may not represent the real in vivo leukemic state (10, 11).

Therefore, it is important to understand the cellular changes accompanying the HTLV-I virus infection in the patients. Here we report that an antigen detected by a monoclonal antibody is selectively expressed on the fresh ATL cells or PBC from healthy HTLV-I carriers but not on ATL cell lines maintained in vitro. In addition, healthy HTLV-I carriers, but not the majority of ATL patients, were found to possess this antigen in plasma. Using thin-layer chromatography plus immunostaining, the antigen recognized by the monoclonal antibody was revealed to be a neutral glycolipid.

MATERIALS AND METHODS

Cells and Sera. PBC from patients and healthy donors were separated by Ficoll-Hypaque density gradient centrifugation. The diagnosis of ATL was made on the basis of clinical features, hematological characteristics (1), and serum antibodies to HTLV-A (6). PBC and sera from healthy HTLV-I virus carriers were kindly provided by Drs. K. Takatsuki and T. Hattori, University of Kumamoto, Kumamoto, Japan. In some experiments, PBC from healthy donors was cultured with Con A (20 µg/ml; Sigma, St. Louis, MO), phytohemagglutinin-P (1:1000; Difco Laboratories, Detroit, MI), or pokeweed mitogen (10 µl/ml; Gibco Oriental, Tokyo, Japan) for 3 days. All in vitro cultures including cell line maintaining were performed with RPMI 1640 medium (Gibco) containing 5—10% fetal calf serum (Gibco).

Production of Monoclonal Antibodies. B10.A(3R) mice were immunized i.p. with 1 x 10^7 lymphocytes (spleen and thymus cells) from B10.A(5R) mice together with 1 x 10^7 Bordetella pertussis vaccine. After the primary immunization, the same amount of lymphocytes from B10.A(5R) without the vaccine was used weekly as a booster (i.p.). Three days after the sixth immunization, spleen cells were removed from sacrificed B10.A(3R) mice and fused with mouse myeloma Ag8.653 according to the standard procedure (12). The supernatants of hybridoma-growing wells were screened for reactivity to Con A-activated human T-lymphocytes by indirect cell surface staining. Hybridoma cells from positive wells were closed by the limiting dilution method.

Cell Surface Staining Analysis. One million cells from various sources to be tested were incubated with 200 µl of hybridoma culture supernatant (about 1 µl/ml of IgM detected by the enzyme-linked immunosorbent assay and another hybridoma culture supernatant, same concentration of IgM, served as a negative control) for 1 h on ice. After washing 3 times, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody (Cappel-Coooper Biomedical, Westchester, PA) for 30 min on ice and analyzed by FACS. Since the total cell populations were studied at different times, it was nearly impossible to correctly evaluate the fluorescent intensity. Thus, for convenience, the percentage positive was determined by measuring the stained cells compared to the control staining as %.

RIA for VJ-41 Antigen in Plasma. Fifty µl of purified mouse monoclonal antibody VJ-41, mouse IgM or BSA (the latter 2 as negative controls) at 50 µg/ml, respectively, were coated in 96-well microtiter soft plates (Flow Laboratories, Inglewood, CA) for 60 min. The plates were then blocked with 1% BSA in phosphate buffered saline (PBS) for 60 min. Fifty µl of serial dilutions of plasma samples were added and allowed to react for 120 min. The wells were washed 3 times with 1%
BSA/PBS and then 50 μl of ¹²⁵I-labeled VJ-41 (at 1 x 10⁶ cpm/ml in 1% BSA/PBS) were added and allowed to react for 120 min. The wells were washed 3 times with 1% BSA/PBS and then twice with 0.5 M NaCl/PBS. The radioactivity bound to microtiter wells was counted by a gamma counter (Yamato, Tokyo, Japan). All the procedures were performed at room temperature.

Thin-Layer Chromatography-Radioimmunostaining of Antigen in Plasma. In order to fractionate the plasma, Sepharose 4B (Pharmacia, Uppsala, Sweden) gel chromatography was performed by monitoring the VJ-41 antigen by RIA as described previously. The fractions containing the VJ-41 antigen (void volume) were pooled and then dialyzed against distilled water for 48 h. These dialytes were lyophilized and subjected to glycolipid extraction. Neutral glycosphingolipids and acidic gangliosides were purified from this dried material according to the method described previously (13). The neutral glycolipid fraction was applied to plastic TLC plates (Macherey-Nagel, Düren, West Germany) which were developed in a solvent system of chloroform:methanol:water (60:35:8). The acidic gangliosides were applied to the plates and developed in a solvent system of chloroform:methanol:0.02% calcium chloride (60:35:8). After the chromatography, immunostaining was performed (14). The TLC plates were soaked overnight in blocking buffer (1% BSA/1% polyvinylpyrrolidone/PBS). The plates were then allowed to react with ¹²⁵I-labeled VJ-41 at 1 x 10⁶ cpm/ml in 3% polyvinylpyrrolidone/PBS. After the reaction, the plates were washed 3 times with 0.1% Tween 20/PBS and then twice with 0.5 M NaCl/PBS. The radioactive band was detected on X-ray film (Fuji Film, Tokyo, Japan) by autoradiography.

RESULTS

Establishment and Cellular Reactivity of VJ-41 Monoclonal Antibody. The initial aim of our hybridoma experiments was to produce mouse monoclonal antibodies which cross-reacted with the human homologous counterpart of the murine lymphocyte suppressor molecule (I-J molecule) (15). This idea came from the fact that some antigen epitopes on the major histocompatibility complex II molecules had already been reported to be cross-reactive as, for example, by using mouse alloimmunized antibodies (16). For the I-J molecule bearing target cells, Con A-activated T-lymphocytes were used (17). One monoclonal antibody, VJ-41, was made by the immunization of B10.A(3R) mice with B10.A(5R) lymphocytes, a standard anti-I-J antibody production protocol, and was selected for its weak reactivity to Con A-activated human T-lymphocytes. It was determined that VJ-41 did not meet the criteria of an anti-I-J antibody by functional experiments. However, during a survey on leukemia cells, VJ-41 was found to react rather strongly with freshly isolated leukemic cells from ATL patients. The isotype of the VJ-41 monoclonal antibody using rabbit anti-mouse isotype specific antibodies was determined to be IgM.

The results of the reactivity of VJ-41 measured by cell surface staining and FACS are shown in Table 1. It should be mentioned that mean fluorescence of the stainings could not be compared to each other correctly since total cell populations were tested at different times. The FACS staining profiles did not always clearly delineate the boundary between the positively and negatively stained cells (Fig. 1). Thus we determined the positive staining percentage by a comparison to the negative control. In humans, normal PBC from more than 20 healthy donors were found to be negative (below 3%). In the FACS fluogram, no change of patterns was observed in comparison to the control staining (data not shown). After stimulation of these PBC with a mitogen, such as Con A, phytohemagglutinin, or pokeweed mitogen, slight reactivity (5-10%) was consistently observed.

Studies of fresh leukemic cells from patients showed that VJ-41 reacted with cells from all the ATL cases (36 patients; Fig. 1) but not with other types of hematological disorders (more than 50 cases). There were 4 exceptions: 2 cases of malignant lymphoma (Fig. 1); one case of acute lymphocytic leukemia; and one case of chronic myelogenous leukemia in acute phase showed positive reactions. Interestingly, even though normal healthy PBC were negative, PBC from some HTLV-I virus carriers were revealed to be positive. In addition, it was found that 2 cases of VJ-41 positive non-ATL (one case of malignant
lymphoma and one case of chronic myelogenous leukemia) were HTLV-A positive (data not shown). It is quite possible that these 2 cases were HTLV-I carriers and that no relationship between the HTLV-I virus and the hematological disorders of these cases existed. Unfortunately the HTLV-A of the other 2 cases of VJ-41 positive non-ATL could not be examined.

In order to investigate the cellular population reactive with VJ-41, 2 dimensional analysis using VJ-41 and other monoclonal antibodies (Leu2a/CD8, Leu3a/CD4, Leu4/CD3, and Leu12) was performed. In 3 cases of ATL patients, VJ-41 reacted with Leu3a positive cells (data not shown), suggesting that at least ATL leukemia cells carry VJ-41 antigen. However, studies using PBC from HTLV-I virus carriers and mitogen activated PBC from healthy donors revealed that VJ-41 also reacted with Leu2a, Leu3a, and Leu12 positive cells. From these data, it was concluded that VJ-41 could not recognize an apparent differentiation antigen on a restricted population of leukocytes. Staining of PBC from autoimmune and chronic inflammatory disease, so far, did not show a distinct positive reaction.

The reactivity of VJ-41 to cultured human cell lines changed in each line (Table 1). Some cell lines were always positive but others were consistently negative. There seemed to be no specificity of the reaction to T-cell lineage. Notably, ATL cell lines which had been established by coculturing cord blood cells with ATL cells (18) and IL-2 dependent cell lines from ATL PBC were all negative (16 lines) (19). MT-1 showed a slightly positive reactivity. However, this MT-1 line was different from the other ATL cell lines in that it was established through culturing PBC from an ATL patient without adding IL-2 (20). It is possible that MT-1 is the cell line that most closely reflects the in vivo ATL leukemic cell conditions. So far as we tested, the staining of the cell lines did not correlate with the cell cycle since all the positive staining in FACS showed single peak patterns.

In mice, normal spleen or thymus cells from various strains including B10.A(5R) showed negative staining. Con A-stimulated splenic T-cells, like human PBC, showed slight reactivity. Interestingly, thymus cells from old (10 months) AKR mice, which frequently produce spontaneous thymomas, were also positive. Some murine leukemia cell lines were positive, but no specific pattern of cell types for positive reactivities was obtained.

To sum up, a study of normal tissues has, so far, shown that VJ-41 did not appear to react with normal cells. Positive reactions were observed in some malignant cell lines from humans and mice in vitro. However, in studies on leukemia cells freshly isolated from patients, VJ-41 was shown to react selectively with ATL cells but not with other hematological disorders. ATL cell lines maintained in vitro were negative. In addition, states predisposed to leukemia (such as PBC from HTLV-I carriers or thymus cells from old AKR mice) seemed to be positive. The antigen recognized by VJ-41 was detected not only on the cell surface but also in the cytoplasm of these positive cells (data not shown).

Detection of VJ-41 Antigen in Plasma. We addressed the question of whether this antigen is also present in the circulating plasma. Solid phase radioimmunoassay was carried out using ATL patient's plasma or plasma from healthy HTLV-I carriers. RIA performed with one HTLV-I carrier's plasma (Fig. 2) clearly showed that only the combination of VJ-41 for coating and 125I-labeled VJ-41 for the tracer enabled us to detect specifically the antigen for the VJ-41 antibody in plasma. Having established the system, plasma from normal donors, ATL patients, and HTLV-I carriers were screened (Fig. 3). Interestingly, 9 of 10 HTLV-I carriers showed higher VJ-41 antigen levels in plasma than did normal donors. On the contrary, the majority of ATL patients showed quite similar antigen levels to normal ones.

Molecular Characterization of VJ-41 Antigen. We next investigated the chemical properties of the antigen molecule recognized with this VJ-41 antibody. Externally or internally labeled proteins using [125I]- or [35S]methionine were precipitated with VJ-41 and analyzed by SDS-PAGE, but no specific band was detected. Immunoblotting of proteins after SDS-PAGE also revealed negative reactivities. These results suggested that the target antigen molecule might not be of a proteinaceous nature.

From the fact that the VJ-41 antigen was detected in the plasma, we tried to fractionate a HTLV-I healthy carrier's plasma using Sephacryl S300 and Sepharose 4B gel chromato-
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tography by monitoring \( A_{280} \) and RIA for VJ-41 antigen. From both columns, the VJ-41 antigen was recovered in the void volume fractions (data not shown). Because there were few proteins in these void volume fractions detected by SDS-PAGE and staining with Coomassie blue (data not shown), it was suggested that VJ-41 antigen could be located within the micelles of lipids. Thus the VJ-41 antigen containing fractions from a Sepharose 4B column were pooled and glycolipids were extracted. The extracted glycolipids were then treated with an alkaline solution (0.5 M NaOH) at 40°C for 30 min followed by 2 days of dialysis against distilled water in order to eliminate the contamination of proteins such as hydrophobic glycopeptides. In the analysis of these glycolipids by TLC followed by immunostaining with \( ^{125}I \)-labeled VJ-41, the antigen was detected in neutral glycolipids but not in the ganglioside fractions (Figs. 4 and 5). Positive immunostaining was also obtained when the sample was treated at 60°C for 1 h before glycolipid extraction, suggesting that the antigen is heat stable (data not shown). Therefore it was concluded that the antigen for VJ-41 in the plasma of healthy HTLV-I carriers is a certain neutral glycolipid with a longer sugar moiety than GA\(_1\) (asialo GM\(_1\)). Several experiments were attempted in order to detect the cellular antigen, but no clear band was observed. We could not completely rule out the possibility that the VJ-41 monoclonal antibody detected a different molecule on the cell only due to the cross-reaction. However, considering the reactivity of the antibody both on the cells and in the plasma associated with HTLV-I, it is reasonable to suppose that VJ-41 recognizes the same glycolipid on the cells. The failure to detect it among the cellular materials could very well be due to technical problems. On the other hand, there is also a possibility that VJ-41 recognizes a carbohydrate antigen both on a glycolipid in the plasma and on a glycoprotein on the cell surface.

DISCUSSION

There is much evidence to support the existence of a strong relationship between HTLV-I virus infection and ATL, but there are still some questions that need to be answered in order to reach a sufficient level of understanding regarding details of the mechanisms of the leukemogenesis of ATL: (a) fresh leukemia cells from ATL patients do not express the HTLV-I gene products such as HTLV-A or p40\(^+\) (6). For the detection of HTLV-I antigen, it is necessary to culture the leukemia cells \textit{in vitro} for a certain period (21). This indicates that the maintenance of the leukemic state \textit{in vivo} does not require the expression of the HTLV-I; (b) it is evident that HTLV-I carries no oncogene and that there is no unique integration site for this virus in the human genome (5, 7). It is therefore suggested that the usual leukemogenesis such as \( v-onc \) transduction (22) or insertional mutagenesis (promoter insertion model) (23) might not play a role in ATL. Transactivation of other genes such as cellular oncogenes, IL-2, or IL-2 receptor by HTLV-I has been thought to be an alternative explanation for this leukemogenesis (24); and (c) many ATL cell lines have been established and found to retain the HTLV-I virus in the genome, but recent studies have suggested that these cells do not represent the real leukemic situation (10, 11). In order to answer these questions, it is important to know the cellular changes caused by \textit{in vivo} infection of the HTLV-I virus as well as the differences between the freshly isolated leukemic cells and ATL cell lines established \textit{in vitro}. In this context, it is interesting that our VJ-41 monoclonal antibody reacts with the HTLV-I carrier PBC and freshly isolated ATL leukemia cells but not with the established ATL cell lines. Because the number of HTLV-I carriers tested was limited, it cannot be stated unequivocally that all carriers possess positive cells. Further study is now being carried out.

It has been recognized recently that some conditions of glycolipid metabolism could be dramatically changed during the malignant state (25, 26). Monoclonal antibodies which react with glycolipid antigens on neuroblastoma cells have been reported (27). The role of aberrantly modified molecules in oncogenesis has also been suggested. The reaction of VJ-41 with HTLV-I infected cells, various \textit{in vitro} cultured malignant cells, and mitogen stimulated cells suggests that there are similar changes in the metabolic pathway. Indeed, direct activations of resting T-lymphocytes by HTLV-I have recently been reported (24), and this observation coincides with the above idea.

The finding that the VJ-41 antigen level was higher in the plasma from healthy HTLV-I carriers than in that from the
majority of ATL patients is rather controversial. It is of course possible that there are 2 different metabolic changes in the course of HTLV-I infection and the leukemogenesis. Further studies will be necessary to uncover the detailed mechanisms of the production of VJ-41 antigen molecules.

There have been some reports on cell surface markers for ATL, such as Leu 3 (OKT4, CD4) (28, 29), IL-2 receptors (Tac) (30), and HTLV-A. Recently, proteins detected by a monoclonal antibody (FTF148) were reported to be expressed on HTLV-I infected cell lines and ATL cells (31). Leu 3 and IL-2 receptor are also detected on normal cells. FTF 148 antigen is not always detected on fresh PBC from ATL patients. HTLV-A is usually detected only after culturing fresh leukemia cells in vitro. So far, to our knowledge, there is no distinctive surface marker for the leukemia cells freshly isolated from ATL patients. For this reason, in clinical applications, this VJ-41 monoclonal antibody may prove to be useful for the diagnosis of ATL.

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