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*J Immunol* 1986; 136:1442-1445;

http://www.jimmunol.org/content/136/4/1442
A monoclonal antibody against LAV GAG precursor: Use for viral protein analysis and antigenic expression in infected cells

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A murine monoclonal antibody (MAb), named C.V.K., was produced after immunization with highly purified and sonicated lymphadenopathy-associated virus (LAV). No monoclonal antibody was observed with intact virus used as immunogen. C.V.K. MAb recognizes an epitope present on the precursor GAG protein of 55 kilodaltons. Western blot analysis and pulse-chase experiments support the interpretation that after p55 cleavage into p25, p18, and p13, only p18 expresses this epitope. C.V.K. MAb selectively stained only LAV-infected lymphocytes. This intracytoplasmic staining appears 3 days after the infection and is correlated with reverse transcriptase activity. Neither membrane immunofluorescence of infected lymphocytes nor neutralizing activity was observed with C.V.K. MAb. These facts suggest that p55 and p18 are not expressed at the cell membrane or on the viral envelope. C.V.K. MAb should prove useful not only in the purification of core proteins but also for detecting infected cells producing the virus in suspension or on histologic sections.

Lymphadenopathy-associated virus (LAV)* is a novel retrovirus that was first isolated in 1983 (1). Its etiologic role in acquired immune deficiency syndrome (AIDS) and related syndromes has been demonstrated by many observations. First, LAV has been almost exclusively isolated from patients presenting the various clinical forms of these syndromes and belonging to all the known “risk” populations (homosexuals, Haitians, Africans, hemophiliacs, i.v. drug abusers, and blood transfusion recipients) (2). Second, LAV antibodies have been detected in significant percentages among patients and healthy people belonging to these “high risk” populations, whereas less than 1% of the general population (as estimated with volunteer blood bank donors) present such antibodies (3). Third, LAV biologic properties, mainly its T4+ lymphocyte tropism and its cytopathic effect, can explain the major immunologic abnormalities noted in AIDS patients (4–6).

Since LAV was first isolated, independent isolates of the same virus, sometimes called human T lymphotropic virus-III (HTLV-III) or AIDS-related virus (ARV), have been obtained throughout the world (7, 8). In addition to the classical gag, pol, and env genes usually found in retroviruses, two other open reading frames, called Q and P, have been described (9). The products of these latter have not yet been characterized. Core proteins are coded for by gag, pol codes for the reverse transcriptase, the protease, and the endonuclease; and env codes for the envelope glycoprotein. Until now, all these proteins have only been described by using reactivity of sera with viral antigens from immunized individuals. The core proteins are 25, 18, and 13 kilodaltons (KD). The envelope glycoprotein has a higher m.w. (110 KD) (10).

We report here the characterization of an anti-LAV monoclonal antibody (MAb) that recognizes an epitope present on the precursor gag protein of 55 KD, which is subsequently cleaved into p18, p25, and p13. After p55 cleavage, only p18 expresses this epitope.

MATERIALS AND METHODS

Mice. Six-week-old BALB/c mice (IFFA-Credo, France) were used for immunization or as source of feeder cells.

Myeloma cell line. P3 × 63 Ag 8.653, a BALB/c nonsecretory plasmacytoma line, was maintained in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; Boehringer Mannheim, West Germany), 1 mM MEM sodium pyruvate (GIBCO) and 2 mM-glutamine (Boehringer Mannheim).

Preparation of antigenic extracts (11). Cellfree supernatants from LAV-producing T lymphocytes were precipitated with 10% polyethyleneglycol (PEG 6000). Then, the virus was banded three times to equilibrium in a 20 to 60% sucrose gradient. After centrifugation at high-speed centrifugation (45,000 × G/20 min), the viral pellet was resuspended in NTE buffer (10 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA), and the protein concentration was adjusted to 1.4 mg/ml. Finally, purified viral particles were treated with ether, and half of the preparation was sonicated in order to expose the core proteins.

Immunization. Female BALB/c mice were inoculated i.p. with 10 μg of antigenic extracts at weekly intervals for 4 wk: two mice received sonicated preparations, and two received nonsonicated preparations. Separate fusions were carried out 3 days after boosting the animals.

Fusion procedure. On the day of fusion, spleen cells were harvested from the immune mice and fused to P3 × 63. Ag 8.653 according to the procedure of Fazekas de St. Groth and Scheidegger (12). Briefly, spleen cells and myeloma cells, at a ratio of 5:1, were mixed together at 37°C in the presence of 50% PEG 4000 (Merck & Co., Inc., Rahway, NJ) and grown in hypoxanthine, aminopterine, and thymidine (HAT medium; Boehringer Mannheim).
supplemented RPMI 1640 medium, with 20% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 5 × 10^{-5} M 2-mercaptoethanol (SIGMA Chemical Co., St. Louis, MO). The 1:10 fusion mixture aliquots were dispensed into 24-well tissue culture Cluster plates (Costar 3524). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and examined by using an inverted microscope for the growth of colonies.

Fourteen days later, culture supernatants were screened for immunoglobulin MAb and antibody reactivity with LAV by enzyme-linked immunosorbent assay (ELISA) methods. Positive colonies were cloned twice by limited diluting on feeder layers of BALB/c thymocytes. Hybrid cell lines were then injected into BALB/c mice primed with pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co.) and grown as ascites tumors. ELISA. ELISA was performed as described (3). Briefly, each supernatant was comparatively tested on both viral preparation and control lysate of normal uninfected lymphocytes. The specifically fixed IgG were revealed by a peroxidase-conjugated goat anti-mouse IgG (Institut Pasteur, France). The enzymatic reaction was carried out with o-phenylenediamine used as substrate.

Isotype analysis. The class and subclass of antibodies present in culture supernatant were determined by gel diffusion analysis, performed by using affinity-purified rabbit anti-mouse IgG1, 2a, 2b, and IgG3 (Institut Pasteur, France).

Western blot analysis (WBA). Pellets of ultracentrifuged supernatants from the LAV-producing T cell acute lymphocytic leukemia line CEM, or cell lysates from infected or uninfected T cell ALL line MOLT/T4 were used as antigen preparations. Viral and control antigens diluted in sample buffer were electrophoresed on 12.5% SDS-polyacrylamide gel (SDS-PAGE). Proteins in the gel were then electrophoretically blotted onto a nitrocellulose filter (10); blots were first incubated with a blocking buffer, BLOTTO (13), for 1 hr at room temperature. MAb or LAV-positive or -negative human control sera were added at a final dilution of 1/50 to 1/200, and the blots were incubated at 4°C overnight. After incubation, the filters were washed three times with buffer (0.3% Tween in PBS) and incubated with 1% normal goat serum for 1 hr. After three more washes, peroxidase-coupled goat anti-mouse or anti-human IgG (1:2000) (Institut Pasteur, France) was added to the strips for 1 hr. They were then washed, and antibody binding was detected by using diaminobenzidine as substrate for the peroxidase reaction.

Radioimmunoprecipitation assay (RIPA) (10). LAV-infected MOLT/T4 cells were pulse labeled with 200 μCi/ml of [35S]cysteine (Amersham: 1400 Ci/mM) for 30 min in cysteine-free medium, then chased in normal cysteine-enriched medium for 1.2 and 4 hr. After the chase period, cells were washed, lysed in NTE buffer containing 1% Triton X-100 (Sigma) and 5% aprotinin (Zymofren: Spera), and the soluble extracts were clarified by a 10-min centrifugation at 10,000 × G. For precipitation, 10 μl of the appropriate MAb, as well as a positive and a negative serum sample, were mixed with the lysates for 16 hr at 4°C. The immune complexes were then bound to protein A-Sepharose (Pharmacia, Upsala, Sweden), washed, and eluted by boiling for 2 min. The immunoprecipitated labeled antigens were analyzed by polyacrylamide gel electrophoresis under reducing conditions (SDS-PAGE) and autoradiography. LAV antigen detection in infected cells. Acetone-fixed cells were incubated for 1 hr at 37°C in a humidified atmosphere, with 1/25 MAb in ascitic form. After three washes in PBS, slides were again incubated for 30 min at 37°C in a humidified atmosphere first with 1/100 biotinylated sheep anti-mouse Ig, and after three more washes in PBS, with 1/100 streptavidin-biotinylated horseradish peroxidase complex (Amersham, U.K.) under the same conditions. Antibody binding was detected by using diaminobenzidine as substrate for the peroxidase reaction, enhanced by the addition of NiCl₂.

Biologic activity. Proliferation of normal human lymphocytes stimulated by PHA, Con A, pokeweed mitogen, or alloantigens, as well as infection of lymphocytes by mature viral particles and fusion of infected and noninfected MOLT/T4 cells, were fully described elsewhere (14–16). For blocking experiments, 1 × 10⁶ cells were incubated with 1/20 diluted ascitic fluid for 60 min at 37°C. After washing, biologic activities were performed as described.

RESULTS

Hybridoma screening. Hybridomas were obtained from the separate fusions of four different mice spleen cells with nonsecretant myeloma cells. Supernatants from 648 wells were screened for Ig production and antibody reactive with LAV by ELISA methods. One strongly positive MAb, named C.V.K., was cloned by limiting dilution on thymocyte feeder layers. Clones were then expanded in vitro and in mouse ascites. The isotype of C.V.K. MAb was determined to be IgG2a by radial diffusion.

Antigen identification. C.V.K. MAb, as well as all its subclones (C.V.K.1, C.V.K.2), specifically recognized proteins of 55, 40, and 18 KD by WBA. The p18 corresponds to the already identified core protein of mature viral particles that is frequently recognized by patients’ sera (10). The p55 and p40 were detected in virus preparation and LAV-infected cell lysates but not in control antigenic lysates of uninfected cells (Fig. 1). They therefore appear to actually be virus-specific proteins. Indeed, p55 appears to be the product of the whole gag gene, and the p40 recognized here might be an intermediate product of the p55 cleavage into p18, p25, and p13. The fact that p18 is barely detectable in LAV-infected cell lysates suggests that such cleavage might occur late during the virus-production cycle.

Pulse-chase experiments strengthened this interpretation (Fig. 2). At the time of [35S]cysteine pulse, only p55 could be detected. One hour after the chase with “cold” cysteine, both p55 and p40 were present; and at 2 hr, p18 appeared in addition. Finally, 4 hr after chase, mostly p18 was observed. Therefore, C.V.K. MAb and its subclones recognize the gag precursor through an epitope

![Figure 1.](http://www.jimmunol.org/) BWA of the proteins recognized by two subclones of C.V.K. MAb [C.V.K.1 and C.V.K.2], Control sera used were as follows: Pos (LAV-specific serum from a patient) and Neg (serum from a healthy LAV-negative individual).

![Figure 2.](http://www.jimmunol.org/) SDS-PAGE analysis of immunoprecipitate of [35S]cysteine pulse-chased MOLT/T4 cells. Lane P: at the end of the pulse; lane 1, 2, 4: respectively 1, 2; and 4 hr after the chase. C.V.K. MAb (A), a positive serum from a LAV-infected individual (H), and a serum from a healthy LAV negative individual (C) were used.
that is conserved on its p18 product.

**Antigen detection in infected cells.** We next determined whether C.V.K. MAb detected LAV antigen expression in infected cells. To this end, PHA-stimulated normal T cells were infected with LAV and cultured in IL 2-supplemented medium as described (1). Sequential reverse transcriptase activity measurement and immunoperoxidase staining of fixed cells were then performed. Samples obtained from parallel uninfected cultures were used comparatively as controls. As shown in Table I, C.V.K. MAb consistently labeled infected cells. This intracytoplasmic staining was detectable as soon as 3 days after infection and disappeared at the time when virus production was no longer detectable in culture supernatant. In contrast, control uninfected cultures were not marked (Fig. 3).

**Biologic activity.** Preincubation of mature viral particles with different concentrations of C.V.K. MAb did not neutralize their capability to infect lymphocytes. In addition, they did not block fusion of infected or noninfected MOLT/T4 cells. Finally, they did not inhibit the proliferation, under optimal conditions, of normal human lymphocytes stimulated by PHA, Con A, pokeweed mitogen, or alloantigens.

**DISCUSSION**

To obtain monoclonal antibodies, we immunized BALB/c mice with two different antigenic preparations. Highly purified virus obtained from supernatants of infected cultured lymphocytes was inactivated by ether treatment, which does not disrupt viral particles. Such preparation was injected in order to obtain antibodies against the protein expressed on the virus membrane, namely the glycoprotein envelope. No MAb was obtained by this procedure. Similarly treated particles were sonicated before injection in order to expose the core proteins: one MAb that recognized an epitope common to p55, p40, and p18 was obtained. These three bands were virus specific, because they were recognized by human LAV-positive sera, but not by sera from healthy controls. Furthermore, when WBA was performed against cellular extracts of uninfected cells in parallel to cellular extracts of infected cells, these three bands were only detected with the latter antigenic preparation. Similarly, C.V.K. MAb did not react with the cellular extracts used as controls in the ELISA. Nevertheless, the reactivity against cellular proteins highly synthetized only by infected cells cannot be excluded.

Detection of three different protein bands by C.V.K. MAb raises a question as to its monoclonality. However, its cloning by limiting dilution led to the obtention of several subclones, all of which gave the same pattern of reactivity in WBA and RIPA. These results suggest that C.V.K. MAb recognizes an epitope shared by the three proteins. The p55 corresponds to the protein coded by the whole gag gene, a precursor that according to sequence data has a predicted m.w. of 55,841 (9). Traduction of gag gene into a precursor further cleaved by protease is common in retroviruses. These results are strengthened by the correlation between the decrease of p55 and the increase of p18 observed in RIPA during pulse-chase experiments. The p40 might correspond to an intermediate product of p55 cleavage.

Use of this MAb for immunoperoxidase staining gave positive results. Intracytoplasmic staining of infected lymphocytes was observed at the time of reverse transcriptase detection in culture supernatants. Probably the detection of p55 and p18 demonstrates the presence of cells actively producing viral particles. Both the absence of membrane staining and the lack of neutralizing activity of this MAb argue against the expression of p55 and p18 at the cell membrane or on the viral envelope.

This MAb will be useful for studying and purifying large amounts of these proteins. At the present time, only LAV antibodies can be easily detected in sera from patients infected with LAV.

**Figure 3.** Viral antigen detection in LAV-infected cells with C.V.K. MAb and immunoperoxidase staining. A. Infected labeled cells. B. Control uninfected cells.
tients with AIDS, related syndromes, or individuals belonging to groups at risk of developing such diseases. This MAb will be useful for detecting infected cells actually producing the virus in suspension or on histologic sections, and perhaps for directly detecting free antigenic particles by immunocapture. Finally, the availability of other MAb directed to different core or envelop proteins will be of great interest in dissecting the immune response of patients to LAV infection, which will perhaps be of prognostic value.

Acknowledgments. We thank N. Durantin, P. Giroud, and E. Brisson for their excellent technical assistance, and M. Labonde and F. Maria for typing the manuscript.

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