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Multiple Expression of Ly-6C and Accumulation of a Ly-6C Pre-mRNA in Activated Macrophages Involved in Rejection of an Allografted Tumor[1]

Osamu Takikawa, Tohru Oku, Naoto Ito,*,[2] Yumiko Ushio, Naoki Yamamoto, Yukio Yoneda, Junko Tsuji, Antonio Sanchez-Bueno, Vladyslav Verkhusha, and Ryotaro Yoshida[3]

*Departments of Cell Biology and *Molecular Biology, Osaka Bioscience Institute, Suita 565, Osaka, Japan*

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We established a monoclonal antibody against a surface antigen of activated macrophages (Møs) involved in the rejection of an allografted mouse tumor, Meth A. Bacteria-elicited Møs also expressed the antigen but resident or inflammatory Møs, or other leukocytes, did not. The antigen was identified as Ly-6C by isolation of cDNA clones encoding it. Two-dimensional immunoblotting revealed that Ly-6C of the Møs exists in multiple forms with a similar size but with different isoelectric points. A Ly-6C pre-mRNA also accumulated abundantly in the Møs. Thus, the multiple expression of Ly-6C and the accumulation of the pre-mRNA are the features of allograft- and bacteria-elicited Møs.

Macrophages (Møs) are functionally and morphologically a quite heterogeneous population (reviewed in Refs. 1 and 2). In allograft rejection, an infiltration of Møs into the graft is always observed histochemically (3, 4). However, the role(s) of Møs in the rejection have not been fully defined (5, 6). This is mainly due to the technical difficulty in harvesting the Møs from the graft. Therefore we developed an allograft model system in which Meth A tumor cells were transplanted into the peritoneal cavity of allogeneic mice (7, 9). Since Meth A cells are an ascites type, the advantage of this system is that we can obtain the Møs infiltrating into the rejection site simply by lavage of the peritoneal cavity (7-10). In this system, we found (11) that the allografted tumor-induced Møs (AIMs) are highly activated, as evidenced by the expression of mRNAs of inducible nitric oxide synthase, interleukin-12, and interferon-γ-inducing factor, all of which are characteristic molecules elaborated by activated Møs (12-14). We also demonstrated that the AIM is highly cytotoxic against Meth A cells *in vitro* (9), suggesting an effector role of AIM in the graft rejection. In fact, the *in vivo* depletion of these Møs suppressed the rejection of the allografted Meth A cells (15).

In this study, we immunized rats with AIM cells to obtain antibody against an AIM-specific surface marker and established K16.5, a mAb that recognizes a surface Ag abundantly expressed on AIMs. The Ag was also expressed on bacteria (BCG)-elicited Møs but not on

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[2] Present address: Division of Genetics, Department of Molecular and Cell Biology, University of California, Life Sciences Addition Bldg. , Berkeley, CA 94720, USA.

[3] To whom correspondence should be addressed at the Department of Cell Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita 565, Osaka, Japan (FAX:81-6-872-4818, e-mail:ryoshida@obi.or.jp).

Abbreviations: Mø, macrophage; Meth A, 3-methylcholanthrene-induced ascites type murine fibrosarcoma; BCG, bacillus Calmett-Guérin; PBS, phosphate-buffered saline; AP, alkaline phosphatase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; RT, reverse transcriptase; FCM, flow cytometry; mAb, monoclonal antibody; Ag, antigen.

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resident Møs, inflammatory Møs, or other leukocytes examined. Our present data reveal that the Ag is a form of Ly-6C, a differentiation Ag (16-18). We also show that a pre-Ly-6C mRNA accumulated in the AIM cells.

MATERIALS AND METHODS

Møs. Male 7-12-week-old and specific pathogen-free C57BL/6 mice (SLC, Hamamastu. Japan) were used for Mø preparation. AIMs were isolated as described previously (9). Resident Møs were obtained by peritoneal lavage of untreated mice. Bacteria-elicited Møs were collected by peritoneal lavage of mice injected *i.p.* with live *Mycobacterium bovis* BCG (10^7 CFU/mouse) 7 days earlier or with 6% (w/v) sodium casein (1 ml/mouse) 4 days earlier. These Møs were purified by discontinuous Percoll (Pharmacia, Uppsala, Sweden) density-gradient centrifugation, and their purity was more than 90% on the basis of morphology.

Preparation of mAbs to AIMs. Two Donryu rats were immunized intrasplenically with AIMs (5×10^5 cells/spleen), and 3 weeks later the rats were given intrasplenic booster injections with the same number of the Møs, as reported by Spitz (19). Three days after the last immunization, the splenocytes were fused with X63.Ag8.653 mouse myeloma cells. Hybridomas producing mAbs, which bound preferentially to AIM but not to casein-Møs, thymocytes, or splenocytes, were screened by cellular ELISA.

Cloning of cDNAs encoding K16.5 Ag. cDNA libraries in pCEV4 (20) were prepared from poly(A) RNA of AIM cells. About 1.7×10^6 independent clones from the oligo(dT)-primed cDNA library of AIM were mixed with 2.8×10^6 clones from a random hexamer-primed cDNA library, and used to transfect COS-7 cells. The enrichment of cDNAs encoding the K16.5 Ag was done as described by Ito *et al.* (21). DNA sequences were determined by DyeDeoxy Terminator Cycle Sequencing with an Applied Biosystem 373A sequencer.

Cellular ELISA and FCM analysis. After fixation with 1% paraformaldehyde in PBS for 10 min on ice, cells were placed in a microplate at 3×10^5 per well in 25 μl of cold PBS; and then 25 μl of 30% (v/v) heat-inactivated rabbit serum/0.1% NaN_3/PBS and 50 μl of K16.5 mAb were added to each well, and incubation was carried out for 10 min on ice. After having been washed twice with cold BSA-PBS (0.5% BSA/0.1% NaN_3/PBS), the cells were treated with AP-conjugated rabbit anti-rat IgM for 10 min on ice in the presence of 10% heat-inactivated rabbit serum and then washed 3 times with BSA-PBS. Finally, they were incubated at 37 ℃ with *p*-nitrophenyl phosphate solution. The amount of product was determined with a microplate reader at 405 nm. FCM analysis of various cells with K16.5 or AL-21 mAb was carried out by the same method as used for the cellular ELISA except that FITC-conjugated rabbit anti-rat IgM was used as the second antibody. A Becton Dickinson FACScan was used for the FCM analysis. FITC or AP-conjugated rabbit anti-rat IgM and control rat IgM were purchased from Zymed, San Francisco, CA. Anti-Ly-6C mAb (AL-21) was obtained from PharMingen, San Diego, CA.

Northern hybridization analysis. Total RNA was isolated from mouse tissues or cells with TRIzol reagent (Gibco BRL), fractionated in agarose gels containing formaldehyde, and transferred to nitrocellulose membranes (Schleicher and Schnell, Keene, NH). As probes, a 0.6-kb PCR product of pK30 cDNA (positions 331-953) or an oligonucleotide concatemer probe (average size: ~0.5 kb) specific for Ly-6C cDNA (positions 255-295) (24) was used. The probe of human G3PDH was purchased from CLONTECH Laboratories, Palo Alto, CA. A Megaprime DNA labeling system (Amersham) was utilized for ^{32}P-labeling of the probes. Hybridization with pK30 or G3PDH probe was carried out at 42 ℃ in 50% formamide/50 mM Na phosphate buffer, pH6.5/5 × SSC/0.3% SDS/5 × Denhardt's reagent/0.01% sonicated salmon sperm DNA, and the membrane was then washed at 55 ℃ with 0.1 × SSC/0.1% SDS. Hybridization with the concatemer Ly-6C probe was performed at 42 ℃ in 6 × SSPE/0.1% SDS/1 × Denhardt's reagent/0.02% tRNA, and the membrane was washed at 65 ℃ with 6 × SSPE/0.1% SDS. Radioactivity was measured with a Fujix BAS 2000 (Fujifilm Co. Tokyo, Japan).

Antiserum to Ly-6C or pK30 protein. Ly-6C peptide of 13 amino acids (AGVPIKD-PNIRER) and pK30 protein peptide of 15 amino acids (AEEGFGENVMSGYLL) were synthesized, conjugated with keyhole limpet hemocyanin or ovalbumin, emulsified by mixing with Freund's Adjuvant, and injected into two New Zealand white rabbits. After three immunizations at intervals of 2-4 weeks, the serum was collected.

Immunoblotting. Expression of Ly-6C or pK30 protein in cells was analyzed by immunoblotting as described previously (10). An authentic pK30 protein of 76 amino acids (LQCYECYGVPIETSCPAVTCRASDGFCIAQNIELIE-GKFPVTYIKKAEEGFGENV-MSGYLLWTRVPLLQIELFLEL) was produced by expression of the pGXT-4T-1 vector (Pharmacia) in *E. coli.*

Two-dimensional immunoblotting. Proteins of cells (3×10^7 cells) solubilized with 0.2 ml of 8 M urea/0.5% Triton X-100/0.4% Pharmalyte (pH 3-10) were firstly subjected to isoelectric focusing in a 4% polyacrylamide gel strip containing Immobilines (Pharmacia) in the pH range of 3-10. The run lasted for 2 h at 500V, 1 h at 1,500V, 1 h at 2,500V, and 8 h at 3,500V at 5 ℃ in a Multiphor II chamber. Proteins separated on the strips were layered onto the top of a SDS-polyacrylamide gel (15%), and subjected to immunoblotting as described previously (10).

RESULTS

From about 5,000 hybridomas prepared by fusing AIM-immunized rat splenocytes with mouse myeloma cells, we selected a hybridoma that secreted a rat IgM mAb, designated as

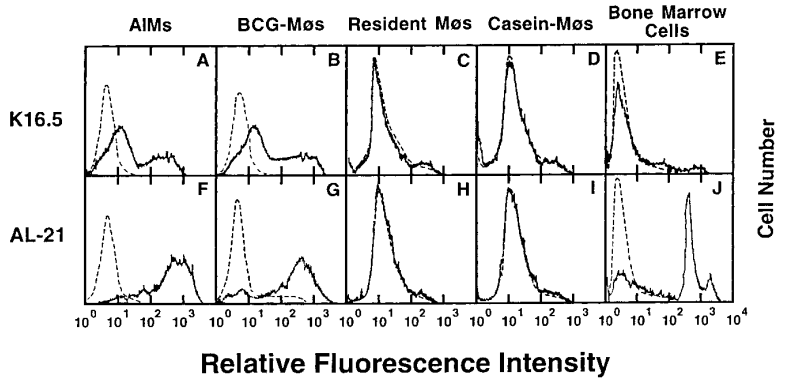

FIG. 1. FCM analysis of Møs and bone marrow cells incubated with K16.5 or AL-21 mAbs. Cells were incubated with K16.5 (A-E) or AL-21 (F-J) mAb, washed, and stained with FITC-labeled anti-rat IgM as described in ''Materials and Methods.'' The dotted lines were obtained by incubating with a control rat IgM. AIM (A, F); BCG-elicited Møs (B, G); resident Møs (C, H); casein-elicited Møs (D, I), and bone marrow cells (E, J).

K16.5, against AIM cells (Fig. 1A). This mAb reacted similarly with BCG-elicited Møs (Fig. 1B) but not with resident Møs (Fig. 1C) or inflammatory Møs such as casein-elicited Møs (Fig. 1D). Also, it did not react significantly with bone marrow cells (Fig. 1E), thymocytes, splenocytes, or lymph node cells (not shown).

To identify the K16.5 Ag, we isolated the cDNAs encoding the Ag. COS-7 cells were transfected with AIM cDNA libraries, and the COS cells expressing K16.5 Ag were enriched by repeated panning and plasmid rescue. After the fourth round of panning, about 10% of the transfected cells expressed the K16.5 Ag. Plasmids recovered from these cells were introduced into *E. coli* by electroporation, and the plasmid inserts from single colonies were analyzed. About 30% of the colonies carried 0.8-kb inserts, and about 5% of them contained 3.0-kb ones. These clones with 0.8-kb or 3.0-kb inserts, upon transfection of COS-7 cells, encoded the K16.5 Ag when examined by FCM. Two clones, pK8 with a 0.8-kb insert and pK30 with a 3.0-kb insert, were selected for sequencing.

The pK8 cDNA sequence was identical to that of Ly-6C, reported by Palfree *et al.* (22). The pK30 cDNA with a length of 2696 bp corresponded to a partially processed form of the Ly-6C gene (23), which included the intron between exons 3 and 4 (Fig. 2). Although this cDNA could produce a Ly-6C variant (pK30 protein) of 76 amino acids if translated usually, the variant was undetectable in AIM or COS-7 cells transfected with pK30 cDNA upon immunoblotting analysis (Fig. 3). Because the Ly-6C variant lacks the conserved hydrophobic

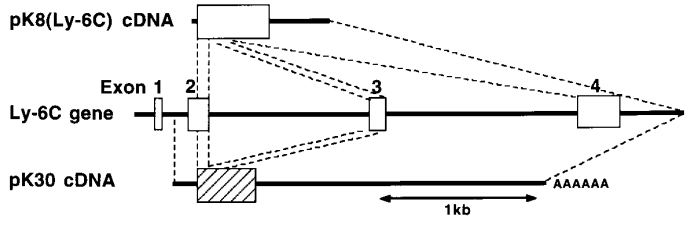

FIG. 2. Comparison of structure of Ly-6C and pK30 cDNAs with that of the Ly-6C gene. The structure of the Ly-6C gene is cited from ref. 23. In the Ly-6C gene, the open boxes represent exons, which are numbered 1 to 4. In the pK8 (Ly-6C) cDNA, the open boxes indicate the coding region. In the pK30 cDNA, the hatched box indicates the region coding for the putative pK30 protein of 76 amino acids.

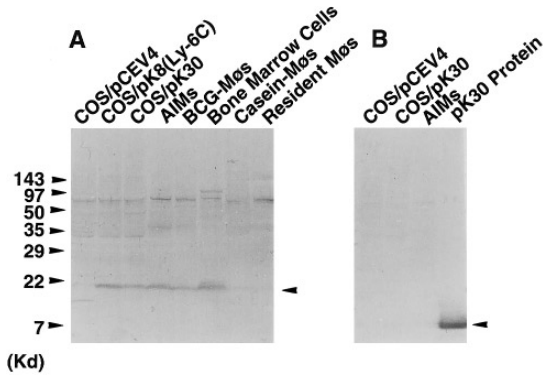

FIG. 3. Immunoblotting analysis of Ly-6C and pK30 protein. Proteins of cells were solubilized, separated on SDS–PAGE (15%), transferred onto nitrocellulose membranes, and blotted with antiserum to Ly-6C (A) or pK30 protein (B) as described in ''Materials and Methods.'' The indicated transfectants of COS-7 cells were prepared by spheroplast fusion as described by Ito *et al.* (21).

C-terminus of 29 amino acids of Ly-6 proteins essential for glycosylphosphatidylinositol-linked anchorage to the plasma membrane (16-18), it would be secreted from AIMs. The variant, however, was undetectable in a 12-h culture medium of AIMs at 3×10^6 cells/ml when assayed by immunoblotting. Rather, we found that the pK30 cDNA transfectants of COS-7 cells produced Ly-6C at the same level as did the Ly-6C (pK8) cDNA transfectants of COS-7 cells (Fig. 3). These results indicate that pK30 cDNA comes from a Ly-6C pre-mRNA and encodes Ly-6C.

Next, we analyzed the expression of both Ly-6C mRNA and the pre-mRNA by Northern blotting using total RNA from AIMs, several types of other Møs, and from various mouse tissues. An oligonucleotide concatemer probe specific for Ly-6C mRNA or a 0.6-kb DNA probe specific for the pre-mRNA was employed. As shown in Fig. 4, a 0.8-kb Ly-6C mRNA was abundantly expressed in AIMs, BCG-elicited Møs, and bone marrow cells. After long

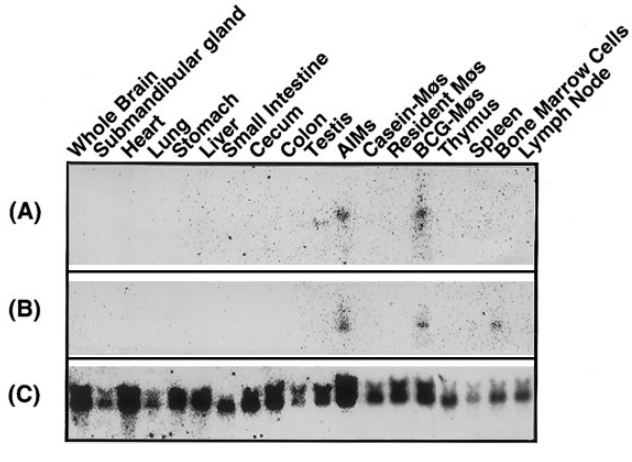

FIG. 4. Northern blot analysis of Ly-6C and pK30 mRNAs. Total RNAs (20 μg per lane) from the indicated sources were Northern blotted as described in ''Materials and Methods.'' The nitrocellulose membrane was first blotted with the pK30 probe (A) and re-hybridized with the Ly-6C probe (B). Finally, the same membrane was hybridized with the G3PDH probe (C).

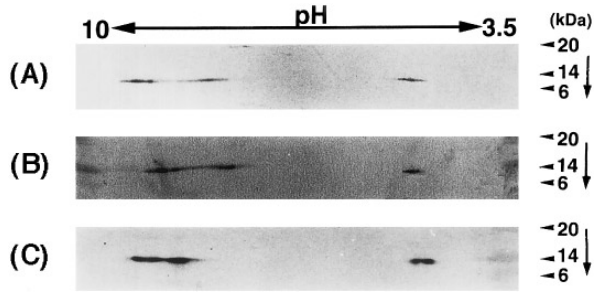

FIG. 5. Two-dimensional/immunoblotting analysis of Ly-6C on AIMs, BCG-elicited Møs, and bone marrow cells. Proteins of cells (3×10^7 cells) were solubilized, subjected to isoelectric focusing, and immunoblotted with antiserum to Ly-6C as described in ''Materials and Methods.'' A, AIMs; B, BCG-Møs; and C, bone marrow cells.

exposure, a faint band of the Ly-6C mRNA was detected in the spleen and lymph nodes (data not shown). On the other hand, a 3.0-kb pre-mRNA was expressed in AIMs and BCG-elicited Møs but was undetectable in other Ly-6C mRNA-positive cells (bone marrow cells) or tissues (the spleen and lymph nodes), even after long exposure (data not shown). Considering the poly(A) tail, the size of the precursor mRNA was almost identical to that of pK30 cDNA, indicating that pK30 cDNA is a full-length cDNA for the mRNA.

The above Northern blotting indicated that Ly-6C was expressed abundantly on BCG-elicited Møs and bone marrow cells in addition to being expressed on AIMs. This was confirmed by immunoblot data obtained with antiserum against Ly-6C (Fig. 3). Furthermore, when the expression of Ly-6C on these cells was examined by FCM using an AL-21 mAb specific for Ly-6C (24), more than 90% of the AIMs (Fig. 1F) or BCG-elicited Møs (Fig. 1G) and about 50% of the bone marrow cells (Fig. 1J) were highly stained; whereas resident Møs (Fig. 1H) or casein-elicited Møs (Fig. 1I) were not significantly stained by the mAb, which results are consistent with those of immunoblotting (Fig. 3) or Northern blot analysis (Fig. 4). Since both K16.5 and AL-21 mAbs are a rat IgM, it is possible to evaluate quantitatively by FCM the amount of each Ag expressed on cells. The extent of staining of AIMs with AL-21 mAb (Fig. 1F) was about 3 times greater than that with K16.5 mAb (Fig. 1A). This indicates that K16.5 recognizes a minor population of Ly-6C molecules expressed on AIMs or BCG-elicited Møs but no Ly-6C molecules on bone marrow cells.

The above FCM analysis suggested a molecular heterogeneity of Ly-6C expressed on AIM, BCG-Møs, and bone marrow cells. To explore this possibility, we analyzed their Ly-6C by 2-dimensional electrophoresis/immunoblotting. Fig. 5 shows that on AIM cells Ly-6C existed in multiple forms having similar molecular weights but different isoelectric points (at least three: one acidic and two basic). Similar multiple forms of Ly-6C were observed with BCG-elicited Mø and bone marrow cells.

DISCUSSION

We established a mAb (K16.5) against AIM cells and isolated two cDNAs (pK8 and pK30) encoding the K16.5 Ag from a cDNA library of AIMs. Both pK8 and pK30 cDNAs encoded Ly-6C, and pK30 cDNA came from a Ly-6C pre-mRNA that accumulated abundantly in the AIM cells. Since splicing of pre-mRNAs takes place in the splicesome and is regulated by many factors including reversible phosphorylation (25, 26), it would be possible that some specific modification(s) of the splicesome results in the accumulation of the pre-Ly-6C mRNA.

Upon FCM, K16.5 mAb was shown to recognize a small part of the Ly-6C population expressed on AIMs and such K16.5 mAb-recognizable ''Ly-6C'' was also present on BCG-

elicited Møs but not on bone marrow cells rich in Ly-6C. In fact, a molecular heterogeneity of Ly-6C was proved: it existed in multiple forms possessing similar *Mr* but different isoelectric points, allowing us to conclude that a minor form(s) of Ly-6C is the K16.5 Ag. Although the precise mechanism operating for the formation of such multiple forms of Ly-6C is presently unknown, it is plausible that a post-translational modification of Ly-6C causes such a diversity. For example, Ly-6C protein is a cysteine-rich protein (9 cysteines per molecule) and thus different combinations of intramolecular disulfide bonds formed might be responsible for generation of the multiple forms. Alternately, it is possible that a microheterogeneity of glycosylation of Ly-6C produces such multiple forms. However, no putative N-linked glycosylation sites (Asn-X-Ser/Thr) have been identified in Ly-6C (22), but the possibility of some O-linked glycosylation has not yet been excluded, as discussed by Gumley *et al.* (18).

The physiological role(s) of Ly-6C expressed on AIMs is unknown, as is the case for Ly-6C expressed on BCG-elicited Møs and other cells including bone marrow cells (16-18), but the recent report of Hatakeyama *et al.* (27) shows that in a murine Mø cell line (J774.1), a protein tyrosine kinase, *fgr,* is associated with Ly-6C. This finding implies that Ly-6C could transduce extracellular signals into cells through tyrosine kinase-dependent phosphorylation of some intracellular protein(s). Actually, Havran *et al.* (28) have shown that Ly-6C expressed on a cytotoxic T-cell clone can transduce a signal required for activation of the cell through an interaction with an anti-Ly-6C mAb. In the cell activation caused by anti-Ly-6C mAb, the mAb is considered to mimic the role of the physiological ligand for Ly-6C, although the ligand has not been identified yet (16-18, 29, 30). In this context, it is of interest to know how the K16.5 or AL-21 mAb affects the function of AIMs. The results indicated that the presence of K16.5 or AL-21 mAb (up to 50 μg/ml) had no inhibitory or stimulatory effect on the cytotoxic activity of AIMs against Meth A cells (Oku *et al.*, unpublished data). These findings, however, do not necessarily mean that Ly-6C on the AIM cell is not involved in the cytotoxic activity. This is because the ability of a mAb to affect the function of cells by binding to its surface Ag is largely dependent on the epitope of the Ag, as shown by a study on T-cell activation by a panel of anti-Thy-1 mAbs, where only some mAbs that bind to an epitope of Thy-1 can activate T-cells (31).

In conclusion, two findings of particular importance in this study are the expression of a Ly-6C form recognized by K16.5 mAb and the accumulation of the Ly-6C pre-mRNA in AIMs. It is unequivocal that these properties are good markers of the AIM cell. Using them, we are currently studying the possible occurrence and involvement of AIMs in other types of allograft rejection.

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