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Generation of Monoclonal Antibodies to the Rabbit Interleukin-2 Receptor α Chain (CD25) and Its Distribution in HTLV-1-transformed Rabbit T Cells

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Rabbits can be infected with human retroviruses such as human T-cell leukemia virus-1 (HTLV-1) and human immunodeficiency virus (HIV), and provide useful animal models to study retroviral diseases such as adult T-cell leukemia and HIV. Previously we have succeeded in generating monoclonal antibodies (mAbs) against rabbit CD4, CD5 and CD11a antigens. To make this animal species more amenable to cellular and molecular studies, we have attempted to extend the panel of mAbs against rabbit CD antigens. Here we report on the generation of three neutralizing mAbs against interleukin-2 receptor α chain (IL-2Rα) (CD25), Kei-α1 (IgG2b), Kei-α2 (IgG2a) and Kei-α3 (IgG1). They specifically recognize the rabbit Mr 55,000 IL-2 binding protein, IL-2Rα, and completely inhibit both high- and low-affinity IL-2 binding to F648b cells that express IL-2Rα as well as IL-2Rβ. The use of mAb Kei-α1 confirmed that the rabbit IL-2Rα is not only a low-affinity IL-2R on its own but also an essential component of high-affinity IL-2R as found in other animal species, and that rabbit activated T cells including HTLV-1-transformed cell lines express high levels of the IL-2Rα. Together with mAbs against various rabbit CD antigens that we reported previously, these neutralizing mAbs to IL-2Rα will be valuable for studies of human retrovirus infections, such as those induced by HTLV-1 or HIV, in rabbits.

Key words: Rabbit — IL-2 receptor α chain — HTLV-1 — T cell

Although the rabbit is an excellent experimental animal species to investigate human retroviral diseases such as those induced by adult T-cell leukemia virus-1 (HTLV-1)[1] or human immunodeficiency virus (HIV),[2-4] the cellular immune system of the rabbit remains to be fully studied. This is mainly due to the fact that only a few monoclonal reagents are available that recognize various differentiation antigens or CD antigens expressed on specific lymphocyte subsets of this species. However, by the use of appropriate immunogens, acquisition of monoclonal antibodies (mAbs) against various rabbit CD antigens is becoming feasible and we have reported successful generation of mAbs against the CD4, CD5 and CD11a antigens.[5] To enable more detailed dissection of various human disease models in this species, we have attempted to generate mAbs against activation antigens in the rabbit, particularly interleukin-2 receptor (IL-2R), whose expression is essential for amplification of immune responses.

The IL-2R consists of at least three IL-2 binding glycoproteins, designated the α chain [IL-2Rα (CD25); Mr 55,000], the β chain (IL-2Rβ; Mr 75,000)[6-8] and the γ chain (IL-2Rγ; Mr 64,000).[9] Our previous studies by the use of a chemical crosslinker with radioiodinated IL-2 indicated that rabbit low-affinity IL-2R is singly expressed IL-2Rα and that high-affinity IL-2R is composed of at least IL-2Rα and IL-2Rβ,[10] as in the human and murine counterparts. However, the rabbit IL-2Rα was identifiable only by the chemical cross-linking method and no monoclonal reagent was available to specifically detect or to functionally block this molecule in the rabbit.

In the present paper we report on the generation and characterization of three mAbs against rabbit IL-2Rα that can completely block low- as well as high-affinity binding of IL-2. High-level expression of IL-2Rα was confirmed in HTLV-1-induced rabbit T cell lines by the use of these mAbs.

MATERIALS AND METHODS

Animals Female Japanese white rabbits of about 2.5 kg in body weight were purchased from Shiraishi Laboratory Animal Farm (Tokyo). Female BALB/c mice of 5 to 7 weeks old were from Shizuoka Laboratory Animal Center (Hamamatsu). All animal experiments in this study were performed according to the guidelines of the Animal Welfare Committee of our institute.

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Cells and cell culture Rabbit T cell lines transformed with HTLV-1, F648b,[11] H446,[12] YR-1 (gift from Dr. M. Hayami, Virus Research Institute, Kyoto University) and RH-2 (gift from Dr. N. Yamamoto, Department of Microbiology, Tokyo Medical and Dental University), and a human T cell line transformed with HTLV-1, HUT102, were cultured in RPMI-1640 containing 10 m*M* HEPES, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10^{-5} 2-mercaptoethanol, 1% (v/v) nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (complete medium). Phytohemagglutinin (PHA) blasts were obtained by incubating rabbit splenocytes with 1% PHA-M (Difco, Detroit, MI) for 5 days.

Cell membrane preparation from F648b was obtained by lysing cells with buffer (pH 7.6, 50 m*M* Tris, 100 m*M* NaCl, 1 m*M* PMSF, and 1 m*M* iodoacetamide) containing 1% NP-40 as described previously.[5] The T and B cells were separated by means of the Ig panning method, as described.[5]

Production of mAbs Female BALB/c mice were immunized by footpad injections of cell membrane preparations of F648b (100 μg/200 μl) at 5- to 7-day intervals. Three days after the fourth booster immunization, splenocytes were fused with mouse myeloma cells (PAI) using polyethyleneglycol 4000 (Merck, Rahway, NJ) as described previously.[5] Ten days after the fusion, hybridoma supernatants were assayed for anti-IL-2R activities by means of IL-2 binding inhibition assay as described below. In order to screen large numbers of hybridomas, pooled supernatants from groups of five hybridomas were tested for the presence of IL-2 binding inhibition activity rather than testing the supernatants individually. When significant inhibitory activity was detected, we examined the five supernatants individually. This enabled us to screen at least 100 to 150 hybridomas at a time by use of the IL-2 binding inhibition assay.

^{125}I-IL-2 binding assay ^{125}I-IL-2 binding assay was performed as detailed elsewhere[13] in the presence or absence of a 400-fold excess of unlabeled IL-2 or 50 μl of hybridoma supernatant. Human recombinant IL-2 (Takeda Pharmaceutical Co., Osaka) was radioiodinated with Enzymobeads (Bio-Rad, Richmond, CA) to a specific activity of 40,000–65,000 cpm/ng. Scatchard plot analysis of ^{125}I-IL-2 binding data was done by using the MS-DOS N88BASIC computer program (provided by Dr. K. Teshigawara, Kyoto University).

Immunoprecipitation and gel electrophoresis F648b cells were radioiodinated by a glucose oxidase/lactoperoxidase method[14] and solubilized in NP-40-containing lysis buffer.[10] Immunoprecipitation was performed by the solid-phase immunoisolation technique (SPIT).[15] The immunoprecipitates were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) on an 8.5% acrylamide gel under reducing and non-reducing conditions.

Flow cytometric analysis Cells (1×10^6) were incubated on ice in a volume of 100 μl with Protein A-purified mAb (5 μg/ml) for 30 min and stained with FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Tago, Burlingame, CA). After washing, the cells were assayed for fluorescence in an EPICS-CS flow cytometer (Coulter, Hialeah, FL).

Assay for IL-2-dependent T cell growth PHA blasts (2×10^4 cells/well) were added to 96-well flat-bottomed plates and cultured for 3 days with IL-2 (1 p*M* to 10 n*M*) in the presence or absence of mAb (50 μg/ml). The T cell growth was measured in terms of incorporation of [^{3}H]thymidine (0.5 μCi/well) (New England Nuclear, Tokyo, 6.7 Ci/mmol; 1 Ci=37 GBq) in 4 h.

RESULTS

Generation and characterization of mAbs against rabbit IL-2Rα In order to generate mAbs against rabbit IL-2Rα, we employed as the immunogen membrane preparations from an HTLV-1-induced rabbit T cell line, F648b, that expresses both IL-2Rα (Mr 55,000) and IL-2Rβ (Mr 75,000) as identified by chemical crosslinking of radiolabeled IL-2.[10] Hybridomas were screened for anti-IL-2R activity by assay for inhibition of ^{125}I-IL-2 binding to F648b cells. Three hybridoma cell lines were isolated and designated Kei-α1 (IgG2b), Kei-α2 (IgG2a) and Kei-α3 (IgG1), respectively. The Kei-α1, Kei-α2 and

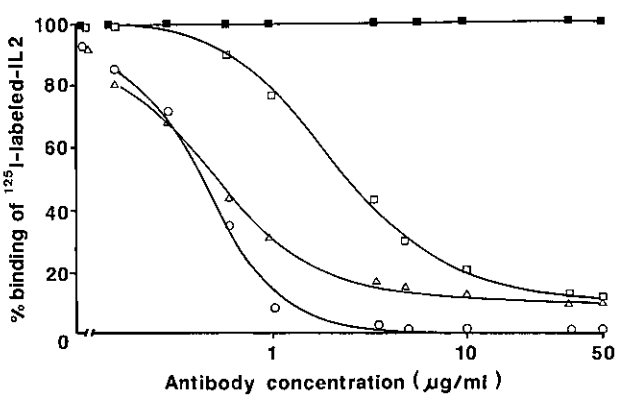

Fig. 1. Inhibition of ^{125}I-IL-2 binding by mAbs Kei-α1, Kei-α2 and Kei-α3. Half a million F648b cells (○, △, □) or HUT102 cells (■) in 50 μl of medium were incubated in the presence or absence of various concentrations of Kei-α1 (○, ■), Kei-α2 (△, ■) or Kei-α3 (□, ■) for 30 min at 4°C. Then, 25 μl of 5 n*M* ^{125}I-IL-2 was added and the cells were incubated for 1 h at 4°C. Cell-bound radioactivity of ^{125}I-IL-2 was measured as described previously[7] and non-specific binding of ^{125}I-IL-2 was determined in the presence of a 400-fold excess of unlabeled IL-2.

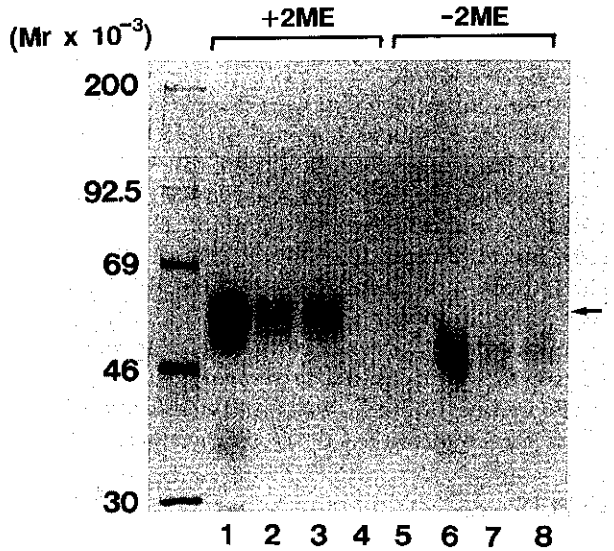

Fig. 2. Immunoprecipitation of the molecule defined by Kei mAbs. Surface radiolabeled F648b cells were solubilized and immunoprecipitated with Kei-α1 (lanes 1 and 6), Kei-α2 (lanes 2 and 7), Kei-α3 (lanes 3 and 8) or a control IgG2a mAb, WT. 1 (lanes 4 and 5). The immunoprecipitates were analyzed on 8.5% acrylamide gel under non-reducing (lanes 5 to 8) or reducing (lanes 1 to 4) conditions.

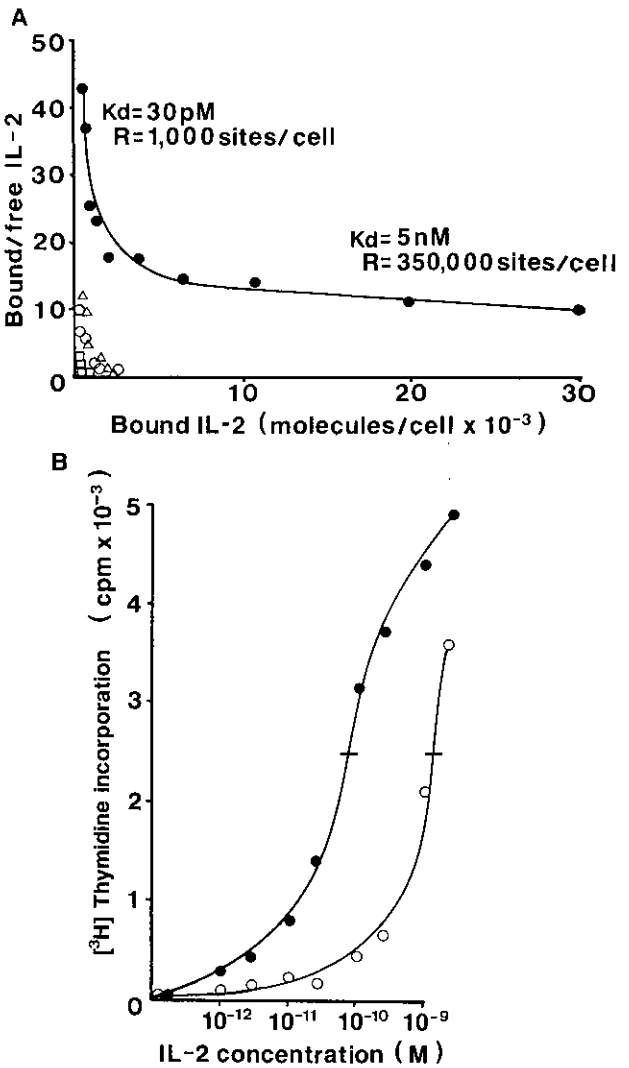

Fig. 3. Effects of Kei mAbs on IL-2 binding to a rabbit T cell line F648b and IL-2-induced cell proliferation of PHA blasts. (A) Scatchard plot analysis of ^{125}I-IL-2 to F648b cells. The binding study was performed in the absence (●) or presence (50 μg/ml final) of Kei-α1 (○), Kei-α2 (△) or Kei-α3 (□). The number of high-affinity sites was 1,000/cell with a K_d of 30 p*M*, and the number of low-affinity sites was 350,000/cell with a K_d of 5 n*M*. (B) Proliferative responses to IL-2. PHA blasts (2×10^4 cells/well) in 200 μl of complete medium were cultured in triplicate for 3 days with various concentrations of IL-2 in the absence (●) or presence (○) of 50 μg/ml of Kei-α1. Cellular proliferation was measured in terms of the incorporation of 0.5 μCi/well of [^{3}H]thymidine during the last 4 h of culture.

Kei-α3 all dose-dependently inhibited ^{125}I-IL-2 binding to F648b cells, but did not inhibit binding to an HTLV-1-induced human T cell line HUT102 that expresses human IL-2Rα and IL-2Rβ (Fig. 1), indicating that the antibodies specifically recognize the IL-2-binding site on rabbit IL-2R. Although these mAbs produced different immunoglobulin isotypes and were derived from independent clones, the binding was completely and reciprocally blocked by companion antibodies in all combinations examined (data not shown), suggesting that these mAbs recognize the same or very closely related epitopes on the IL-2-binding site of the rabbit IL-2R. When F648b cell extracts were immunoprecipitated by mAbs Kei-α1, Kei-α2 and Kei-α3, it was found that all precipitates produced a single band of about Mr 55,000 or Mr 50,000 on SDS-PAGE gel under reducing or non-reducing conditions, respectively (Fig. 2), consistent with the presence of intrachain disulfide bond(s). A similar observation has been made in human IL-2Rα.[16] These results indicate that the Kei mAbs recognize an IL-2-binding epitope of rabbit IL-2Rα.

Analysis of IL-2 binding to a rabbit T cell line, F648b, and effects of mAbs on the high- and low-affinity IL-2 binding and IL-2-induced T cell growth The mode of IL-2 binding to a rabbit T cell line, F648b, was examined in detail by the use of Scatchard plot analysis of radiolabeled IL-2 binding in the presence or absence of Kei mAbs. As shown in Fig. 3A, F648b expressed both high- and low-affinity IL-2R. The number of high-affinity bind-

ing sites was 1,000 per cell with a K_d of 30 p*M*, and the number of low-affinity binding sites was 3.5×10^5 per cell with a K_d of 5 n*M*. Previous studies indicated that the low-affinity receptor is IL-2Rα and that the high-affinity IL-2R consists of at least two receptor subunits, IL-2Rα and IL-2Rβ in human,[13] mouse[17] and rabbit.[10] Consistent with this, both high- and low-affinity binding sites present on F648b were completely abrogated by anti-rabbit IL-2Rα mAbs Kei-α1, Kei-α2 and Kei-α3.

We then examined whether these mAbs inhibit the IL-2-dependent T cell growth. Rabbit PHA blasts were cultured with a wide range of IL-2 concentrations (1 p*M* to 10 n*M*) in the presence or absence of Kei-α1 (50 μg/ml) (Fig. 3B). In the absence of mAb Kei-α1, the IL-2 concentration required for a half-maximal [^{3}H]thymidine incorporation was 35 p*M*, indicating that IL-2 utilized high-affinity receptor. In the presence of Kei-α1, the cell growth curve was clearly shifted to the right and the IL-2 concentration for a half-maximal response was increased to 1.0 n*M*, indicating that IL-2 selectively bound to the intermediate-affinity receptor IL-2Rβ, subsequent to blocking of the IL-2Rα by mAb Kei-α1. Kei-α2 and Kei-α3 showed almost identical inhibition patterns (data not shown). Similar inhibitory effects were observed with anti-IL-2Rα antibodies in human[18] and mouse.[19]

Cellular distribution of the rabbit IL-2Rα as revealed by staining with anti-IL-2Rα mAb Kei-α1 In humans, the IL-2Rα is not expressed in resting T cells but is induced rapidly following activation with antigen or mitogen.[20] In addition, IL-2Rα is expressed abundantly in HTLV-1-infected human T cell lines.[21] We therefore examined the expression of IL-2Rα in rabbit T cells and several HTLV-1-infected rabbit T cell lines by flow cytometry using Kei-α1 antibody (Fig. 4). Although only very small proportions (<1%) of T and B cells obtained from peripheral blood were positively stained, a majority of PHA blasts showed intense staining with Kei-α1. Among HTLV-1-transformed rabbit cell lines, both F648b and YR-1 were even more brightly stained than PHA blasts, whereas RH-2 showed a similar staining intensity to PHA blasts. In contrast, H446 showed only a dull staining, but the fluorescence profile was clearly shifted to the right compared to that of the negative control, indicating that a great majority of H446 cells expressed small but significant numbers of IL-2Rα on the cell surface.

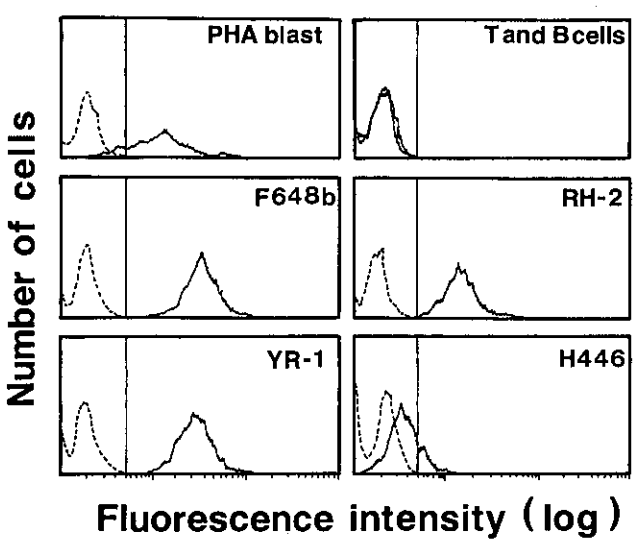

Fig. 4. Flow cytometric analysis of various types of rabbit cells stained with mAb Kei-α1. Cells were first incubated with Kei-α1, then washed and stained with FITC-conjugated $F(ab')_2$ goat anti-mouse Ig. The immunofluorescence was analyzed on an EPICS-CS flow cytometer.

DISCUSSION

We report herein that rabbit IL-2Rα chain (CD25) can now be detected by three neutralizing mAbs, designated Kei-α1, Kei-α2 and Kei-α3. Although all the Kei antibodies gave comparable staining patterns when examined by immunofluorescence staining, Kei-α2 and Kei-α3 were not so efficient as Kei-α1 in immunoprecipitating the IL-2Rα chain, possibly due to their weaker affinities.

We have previously demonstrated that the rabbit high-affinity IL-2R consist of at least two polypeptide chains, IL-2Rα and IL-2Rβ, and that a third component (25 kDa protein) may exist in the high-affinity IL-2R complex.[10] The Kei mAbs generated in this study recognize neither IL-2Rβ nor the 25 kDa protein but specifically recognize IL-2Rα. The use of these mAbs confirmed that IL-2Rα is not only a low-affinity receptor but also an essential component of high-affinity receptor in the rabbit, since these antibodies blocked both high- and low-affinity IL-2 binding and also cell proliferation of rabbit PHA blasts induced by picomolar concentrations of IL-2.

At least two factors probably contributed to the successful generation of the three neutralizing mAbs against rabbit IL-2Rα. First is the use of an HTLV-1-transformed rabbit T cell line that expresses high levels of IL-2Rα. Second, the use of the IL-2 binding inhibition assay also contributed to successful detection of neutralizing mAbs. Although screening by virtue of antibody staining is easy and rapid, it is not particularly useful for identifying neutralizing antibodies that can block the function of the antigen recognized. Therefore, we had to identify blocking mAbs by performing laborious IL-2 binding inhibition assay rather than antibody staining assay by flow cytometry or ELISA.

Although most HTLV-1-transformed rabbit T cell lines were found to express large numbers of IL-2Rα as judged by strong immunofluorescence staining with Kei mAbs, H446 exhibited only a meager fluorescence. Previously we demonstrated by radiolabeled IL-2 binding that this cell line also possesses large numbers of low-affinity IL-2 binding sites[10] that can be completely abrogated by Kei-α mAb, as is the case in other HTLV-1-transformed rabbit cell lines. At the same time H446 was found to release large amounts of soluble IL-2Rα into the culture supernatant (Y. Yamamura *et al.*, unpublished), which may have affected antibody staining of this cell line. The discrepancy observed between the number of binding sites for antibody and that for IL-2 requires further investigation.

These mAbs are a new addition to the panel of mAbs against various rabbit cell surface molecules, particularly those homologous to human CD antigens, and provide useful tools for cellular and molecular analyses of experimentally induced HTLV-1 and HIV infections in the rabbit. We have already generated mAbs against rabbit CD4, CD5 and CD11a.[5] Wilkinson and his associates have generated mAbs against rabbit CD18 (personal communication), CD43 and CD58.[22]

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