

A Monoclonal Antibody against Dolphin Lymphocytes (6E9) which Recognizes Bovine MHC Class II Antigens

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ABSTRACT. A monoclonal antibody, 6E9 established from mice injected with dolphin peripheral blood lymphocytes (PBLs) was characterized. In addition to its reactivity against 89.4% of dolphin PBLs, 6E9 reacted with 33.1% of bovine PBLs of which 22% were CD5⁺, 11.1% were CD5⁻. 6E9 recognized a 34 kD protein on the surface of dolphin and bovine PBLs. Analysis of the protein's N-terminal amino acid sequence indicated that 6E9 recognizes bovine major histocompatibility complex (MHC) class II antigens. These results suggested that 6E9 recognized MHC class II antigens on bovine PBLs. As we have already produced an anti-dolphin MHC class I monoclonal antibody, analysis of immune system using these monoclonal antibodies will advance our understanding of the evolution of the mammalian immune system.—**KEY WORDS:** dolphin, MHC class II antigen, monoclonal antibody.

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Dolphins are very interesting mammals because they have returned to the sea from the land, after acquiring pulmonary respiration system. We are attempting to characterize their immune functions and as the first step towards this goal, we have produced monoclonal antibodies (mAbs) against dolphin lymphocytes.

The use of mAbs that are reactive with cell surface antigens has led to an understanding of the functions of lymphocyte subsets and the biochemical characterization of the molecules with which the antibodies react. The best characterized species include mouse, rat and human, and mAbs that distinguish T and B lymphocytes and subset of T lymphocytes which function as helper or suppressor-cytotoxic cells have been produced. Recent publications have described mAbs that recognize the surface antigens of bovine T lymphocytes. These mAbs recognize the CD2 [3], CD3 [4], CD4 [1], CD5 [7], CD8 [5] and other antigens [10]. In the case of the dolphin, mAbs against human lymphocytes have been analyzed for the cross reactivity to dolphin lymphocytes [2, 11]. However, no mAb have previously been developed against dolphin lymphocytes.

MAbs that recognize dolphin lymphocytes, are needed for the analysis of the dolphin immune system. In this report, we describe the production and characterization of a mAb that recognizes a surface antigen on dolphin and bovine lymphocytes. The antigen recognized by the 6E9 is one of the bovine major histocompatibility complex (MHC) class II antigens.

MATERIALS AND METHODS

Animals and blood samples: Bottlenosed dolphins (*Tursiops Truncatus*) were reared and fed at Minamichita Beachland Aquarium, Okuda, Aichiken, Japan. Dolphin blood was drawn from the vascular system of flukes into plastic tubes containing heparin. Six-week-old male BALB/c

mice were purchased from Clea Japan Inc. (Tokyo, Japan). Cows were maintained at Nihon University.

Production of 6E9 mAb: BALB/c mice were immunized with dolphin peripheral blood lymphocytes (PBLs) isolated by the Ficoll-Paque gradient (1.077) centrifugation method. The immunization schedule consisted of intraperitoneal injections of live 10^7 cells at 2-month intervals. After three injections, splenocytes from immune mice were fused with P3-X63-Ag8.653 myeloma cells [6]. The screening of hybridoma supernatants was based on the reactivity to dolphin PBLs, as analyzed by immunofluorescence staining. A hybridoma, termed 6E9 selected by this screening procedure, was further subcloned by limiting dilution and was cultured in RPMI 1640 (Gibco, Grand Island, New York) containing 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, 1% nonessential amino acids, 1% Na-pyruvate, and 5×10^{-5} M 2-mercaptoethanol (ME), referred to as complete medium.

Immunofluorescence tests: For immunofluorescence staining, cells were incubated at 4°C for 30 min with 30 μ l of hybridoma supernatant. The cells were then washed with cold PBS (pH 7.2) containing 2% heat-inactivated newborn calf serum (NCS) and 0.05% NaN₃, and mixed with 10 μ l of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig mAb (Dakopatts, Copenhagen, Denmark) diluted in PBS containing 2% NCS and 0.05% NaN₃. The expression of the 6E9 surface antigens on bovine PBLs was compared with that of anti-bovine CD4 (CC30), CD5 (CC17), and CD8 (CC63) antibodies using two-color immunofluorescence tests. Namely, cells were stained with these mAbs followed by FITC-conjugated rabbit anti-mouse Ig mAb, and then, cells were stained with biotin-conjugated 6E9 and then visualized with phycoerythrin (PE)-conjugated avidin (Caltag Laboratories, San Francisco, CA). Background staining was assessed using FITC-conjugated anti-mouse Ig mAb and PE-conjugated avidin. The

fluorescence-positive cells were analyzed with a fluorescence activated cell sorter (FACScan; Becton-Dickinson, Mountain View, CA).

Biochemical characterization of the molecule recognized 6E9: PBLs were washed three times in cold PBS, and then the surface of the cells was biotinylated for 40 min at room temperature with 0.1 mg/10⁷ cells/ml N-hydroxysulfosuccinimide (NHS)-LC-biotin (Pierce, Rockford, Illinois) in HEPES/saline pH 8.0 [8]. After being washed in cold RPMI 1640, the cells were resuspended in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, and 500 µg/ml benzamide in 20 mM Tris-HCl pH 7.5 for 30 min at 4°C. Lysates were percolated twice with mouse Ig coupled to protein G-Sepharose (Amersham Les Ulis, France) at 4°C for 3 hr. Subsequently, lysates were immunoprecipitated with purified 6E9 and purified mouse IgG₁ covalently coupled to protein G-Sepharose at 4°C for 12 hr. Immunoprecipitates were washed five times in 100 µl of lysis buffer and then resuspended in 30 µl of sample buffer and applied to a 10% polyacrylamide gel in a mini-slab gel system (Bio-Rad, Richmond, CA). After electrophoresis, protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a transblot cell (Bio-Rad). Nonspecific protein binding was blocked by a 3-hr incubation of the membrane in 1% w/v bovine serum albumin (BSA) in PBS. The membrane was incubated for 1 hr at room temperature with a 1:2,000 v/v dilution of horseradish-peroxidase-coupled streptavidin (Amersham International, Arlington Heights, IL) in PBS containing 0.05% Tween 20 and 1% BSA. The membrane was washed three times and developed by a chemiluminescence procedure according to the instructions provided by the manufacturer (ECL Western Blotting Detection System, Amersham). N-terminal amino acid sequence was requested for Takara Shuzo Co., Ltd.

RESULTS

Establishment of a mAb against dolphin lymphocytes: One mAb, 6E9 (IgG₁) showed reactivity against dolphin lymphocytes as well as bovine lymphocytes (Fig. 1). 6E9 was highly reactive with 89.4% of dolphin lymphocytes. However, its reactivity against bovine lymphocytes was limited to 33.1%.

Analysis of cell surface markers: Bovine PBLs were stained by anti-bovine CD5 mAb (81.5%), anti-bovine CD4 mAb (25.5%) and anti-bovine CD8 mAb (28.5%). In two color analysis, it was found that 6E9-positive cells expressed CD4 (3.6%) and CD8 (4.6%) antigens. Also, 11.1% of PBLs were 6E9⁺CD5⁻, and 22% of PBLs were 6E9⁺CD5⁺ (Fig. 2).

Biochemical characterization of molecules detected by 6E9: Under reducing conditions 6E9 precipitated a protein of approximately 34 kD from both biotin-labeled dolphin and bovine PBLs (Fig. 3). N-terminal amino acid sequence of the precipitated protein of bovine PBLs was found to be

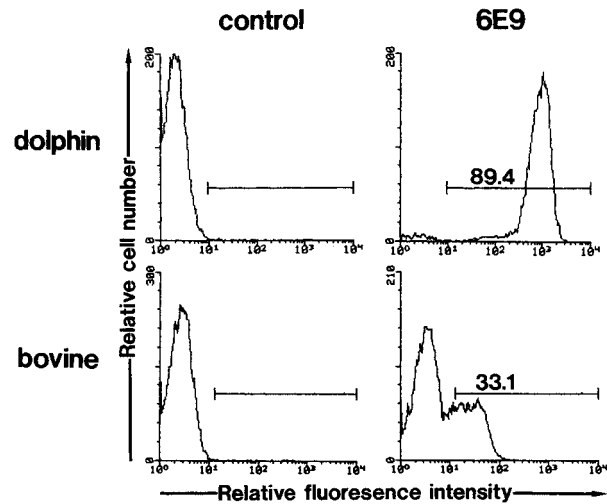


Fig. 1. Reactivity of 6E9 with dolphin and bovine PBLs. FITC-conjugated 6E9 was used in the reactivity tests. Background staining was assessed using only FITC-conjugated anti-mouse Ig mAb.

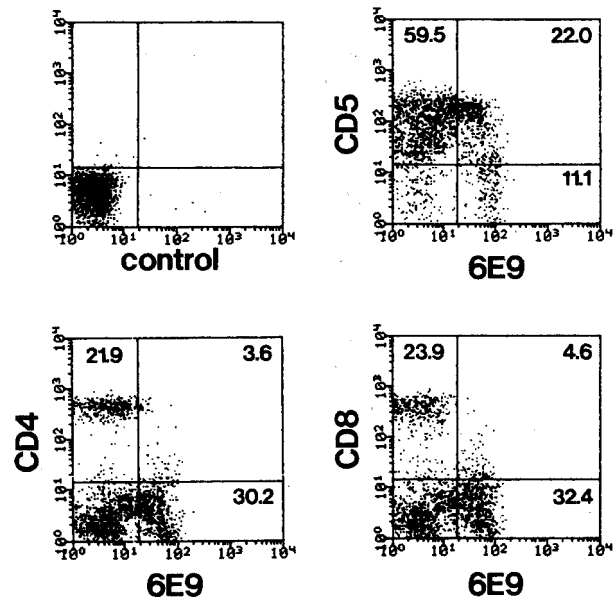


Fig. 2. Two-color staining for 6E9 and CD5, for 6E9 and CD4, and for 6E9 and CD8 in bovine PBLs. Background staining was assessed using FITC-conjugated anti-mouse Ig mAb and PE-conjugated avidin.

similar to those of human MHC class II DP molecules.

DISCUSSION

Dolphins belong to one of only two orders (Order Cetacea and Order Sirenia) of mammals to have left the land to adapt to life in a totally aquatic environment. Consequently, they have features that differ from land mammals. The purpose of this study was to investigate aspects of dolphin immune system.

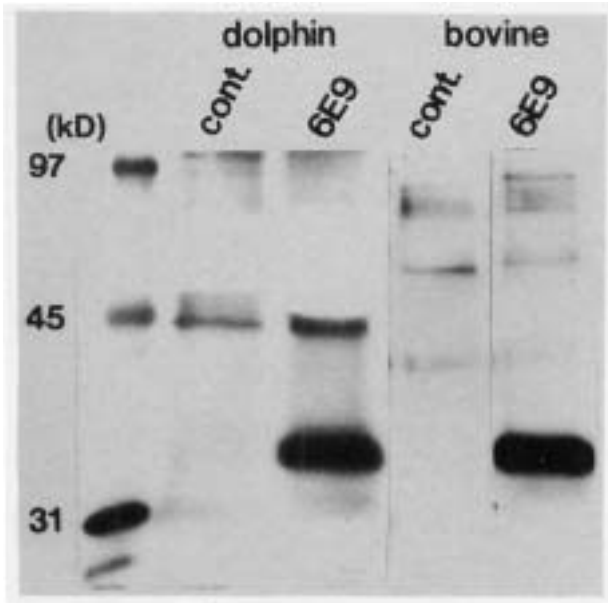


Fig. 3. Immunoprecipitation analysis of surface antigens on dolphin and bovine PBLs using 6E9. Cells were labeled with biotin, lysed in 1% NP-40 and immunoprecipitated with purified mouse IgG₁ (control) or purified 6E9. Samples were analyzed by SDS-PAGE using a 10% acrylamide gel in the presence of 5% 2-ME. 6E9 precipitated a 34 kD molecule from dolphin PBLs and from bovine PBLs.

The present study describes the production and characterization of 6E9. 6E9 reacts not only with dolphin lymphocytes but also with bovine lymphocytes. In bovine lymphocytes, 6E9-positive cells expressed CD4 (3.6%) and CD8 (4.6%) antigens. Also, 11.1% of PBLs were 6E9+CD5⁻, and 22% of PBLs were 6E9+CD5⁺. In immunoprecipitation experiments 6E9 recognized the bovine MHC class II molecule.

Bovine MHC class II molecules are expressed on the surface of bovine B lymphocytes, activated T lymphocytes and monocytes [7]. B lymphocytes that are identified with anti-bovine Ig accounting for 10–30% of bovine PBLs, do not express CD5 [7]. However, plasma cells, a type of B lymphocyte, do express CD5. Monocytes were excluded from the FACS analysis by gating based on size and granularity. Since 6E9 reacts to both CD5 positive and negative bovine PBL, 6E9 seems to recognize MHC class II molecules expressed on the surface of lymphocytes.

On the other hand, 6E9 reacted with approximately 90% of dolphin PBLs. However, immunostaining with dolphin Ig-specific antibodies has shown that Ig-bearing B cells were accounted for only 10–15% of PBLs [11]. Furthermore, it has been reported that a monoclonal antibody against human MHC class II molecules reacts with 90–99% of dolphin PBLs [11]. In humans, T lymphocytes usually do not express class II molecules, but they become class II⁺ when

activated [9]. Therefore, it is possible that 6E9 recognizes not only B lymphocytes but also T lymphocytes of dolphin PBLs.

As we have already produced an anti-dolphin MHC class I monoclonal antibody (impress), analysis of immune system using these monoclonal antibodies will advance our understanding of the evolution of the mammalian immune system.

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