Immunology 1998 **94** 207–212

Characterization of a monoclonal antibody that recognizes a lymphocyte surface antigen for the cetacean homologue to CD45R

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SUMMARY

As part of our current efforts to develop assays and reagents to study the immune system of marine mammals, and in view of the effort currently made to develop monoclonal antibodies to cell surface proteins of lymphocyte subsets in different species, the present paper reports on the characterization of a monoclonal antibody against the homologue of CD45R on cetacean lymphocytes. The specificity of this antibody has been characterized on the basis of immunoprecipitation of the antigen it recognized, immunoperoxidase staining on cetacean lymph node and thymus sections, as well as one and two-colour flow cytometric analysis of cetacean peripheral blood mononuclear cells and single-cell suspensions of thymus, lymph node and spleen. Anti-cetacean CD45R (F21.H) immunoprecipitated proteins of 180, 200 and 220×10^3 MW, with the 180×10^3 MW form being predominantly expressed on T cells and the 220×10^3 MW form expressed predominantly on B cells and thymocytes. F21.H labelled all B cells and a proportion of T cells on single-cell suspensions of spleen cells. $CD45R^-$ killer whale peripheral blood lymphocytes expressed a higher density of CD2 than $CD45R^+$, a characteristic of memory T cells. Killer whale T lymphocytes also lost the expression of CD45R upon activation with concanavalin A (Con A) and phytohaemagglutinin (PHA). This is the first report of a monoclonal antibody to CD45R in cetaceans, and this antibody is foreseen as a possible valuable diagnostic and research tool to assess immune functions of captive and wild cetaceans as part of the evaluation of their health status.

INTRODUCTION

Monoclonal antibodies (mAb) which identify subpopulations of peripheral blood leucocytes are essential for investigation of the role of these cells in the pathogenesis and regulation of disease.[1] Although cross-reactivity of mAb between phylogenically relatively distant species has sometimes been observed,[2] a better understanding of immune function and possible eventual therapeutic use of those monoclonal antibodies call for species specific or closely related species cross-reactive reagents. The effort devoted to the development of mAb against leucocyte cell surface markers is best reflected by the numerous symposia recently held to characterize the advances in different domestic species.[3–6]

CD45 or common leucocyte antigen is a family of highly glycosylated proteins expressed only on the surface of nucleated cells of haemopoietic origin.[7] Different isoforms of the molecule have varying molecular weight and are expressed on different cell types as the consequence of the differential use of three exons designated A, B and C.[8] CD45 was shown to have protein tyrosine phosphatase activity[9] and to play an important role in B-cell[10] as well as T-cell[11] activation, and these findings were confirmed by the use of transgenic mice.[12]

As part of our current efforts to develop assays and reagents to study the immune system of marine mammals, the present paper reports on the characterization of a mAb against surface proteins of cetacean leucocytes developed in our laboratory. This homologue to CD45R is the first report of monoclonal antibodies to this cell surface protein of leucocytes in cetaceans. This antibody is foreseen as a possible valuable diagnostic and research tool to assess immune functions of captive and wild cetaceans as part of the evaluation of their health status.

Received 10 December 1997; revised 30 January 1998; accepted 30 January 1998.

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MATERIALS AND METHODS

Monoclonal antibodies (mAb)

mAb against cetacean CD2 (F21.I) and CD21 (F21.F) have previously been produced and characterized in our laboratory.[13]

Production of mAb

BALB/c mice were immunized with 2×10^6 bottlenose dolphin mononuclear leucocytes intraperitoneally at 3-week intervals. mAb were produced as previously described,[14] screened by flow cytometry and cloned by limiting dilution. Antibody isotype was determined by flow cytometry using isotype-specific, fluorescein isothiocyanate (FITC) conjugated reagents (Zymed Laboratories Inc., South San Francisco, CA).

Ascitic fluid was purified using Bakerbond ABX mixed-function ion-exchange matrix (J. T. Baker Inc., Philipsburg, NJ). Purified mAb were directly labelled with FITC (Bethyl Laboratories Inc., Montgomery TX) for double labelling.

Cell and tissue preparation

Cells for this study were from different sources. Blood samples from different species of healthy cetaceans kept in captivity, as well as a few beached animals in transit for rehabilitation purposes, were received from Sea World Parks and were cool after overnight transportation. Killer whale mononuclear cells were isolated from peripheral blood by centrifugation of the buffy coat over Isolymph (Gallard-Schlesinger, Norway) for 30 min at 900 ***g***. Mesenteric lymph node, spleen and thymus were collected from a freshly dead wild common dolphin (*Delphinus delphis*) that was stranded alive and died shortly afterwards at San Pedro Marine Mammal Center (California), and a captive bottlenose dolphin (*Tursiops truncatus*) that died in a Sea World Park. Shortly after death, the tissues were transferred to ice-cold RPMI-1640 with 10% fetal calf serum. Single-cell suspensions were obtained by gently dissociating the lymphoid organs with two pairs of forceps. Cells were cryopreserved in fetal calf serum with 10% DMSO. In all cases, cells were washed in phosphate-buffered saline (PBS), without Ca^{2+} or Mg^{2+}, before use. Lymphoid tissues were collected after the death of cetaceans, which were either kept in captivity or were stranded and transiently taken care of at Mote Marine Laboratory (Florida) and NOSC (California), were snap frozen in O. C. T. Compound (Miles, Elkhart, IN).[14]

Separation of T and B lymphocytes

Bottlenose dolphin splenocytes were separated into B and T lymphocytes using the Mini-MACS magnetic separation system (Miltenyi Biotec Inc., Sunnyvale CA) as per the manufacturer's instructions. Briefly, cells were incubated with an anti-cetacean CD21 (F21.F) mAb followed by incubation with goat anti-mouse immunoglobulin G (IgG) coupled to magnetic microbeads. The cells were then separated using columns on their magnetic holders.

Immunoprecipitations and PAGE

Freshly isolated cells as well as separated B and T lymphocytes were used as antigen sources for immunoprecipitations. Leucocytes were labelled with sulpho-NHS-biotin (Pierce, Rockford, IL), as previously described.[14] Cellular proteins were solubilized with lysis buffer (LB) containing 1·5% w/v Briji 99 and 0·5% Briji 96.[14]

Purified mAb were bound to agarose beads (affigel-10 active ester agarose, Bio-Rad, Richmond, CA), as per the manufacturer's instructions, at a concentration of 20 mg protein/ml packed volume. Labelled cell lysates were incubated with immunoglobulin-coated beads overnight at 4° or for 2 h at room temperature. Beads were pelleted and washed repeatedly in LB. Precipitated proteins, removed from the solid support by incubation with sample application buffer (containing 2-mercaptoethanol), were analysed by discontinuous SDS-PAGE[15] along with biotinylated and non-biotinylated molecular weight standards. Proteins were transferred to Immobilon P (Millipore, South San Francisco, CA), membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0·5% Tween-20, and probed with streptavidin-horseradish peroxidase (Zymed Laboratories Inc.). Membranes were developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL), according to the manufacturer's instructions, and light emission was recorded on Hyperfilm-ECI (Amersham Corp.).

Immunofluorescence staining and flow cytometric analysis

Cell preparations or whole blood samples were analysed by flow cytometry as previously described.[14] Briefly, 1×10^6 leucocytes were blocked with 5% goat serum and stained with mAb indirectly with $F(ab')_2$ anti-mouse (anti-Ms) IgG (H+L) [FITC-conjugated; Caltag, South San Francisco, CA]. Non-specific binding of immunoglobulin to cells was determined by indirect FITC labelling of irrelevant antibody. For double labelling, cells were first incubated with a non-labelled mouse anti-cetacean mAb, then incubated with a phycoerythrin (PE)-labelled goat anti-mouse antibody, and a third incubation was performed with a FITC-conjugated mouse anti-cetacean antibody. In all cases, incubations were performed at 4° in the dark for 30 min. Cells were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Five to ten thousand events were collected using a gate to include only mononuclear cells identified by forward and side light-scatter characteristics. Analysis was performed using gates that were adjusted according to proper controls.

Immunohistochemistry

Diluted hybridoma tissue culture supernatants were applied to frozen sections of common dolphin (*Delphinus delphis*) lymph nodes and thymus. Antibody binding was detected using biotinylated anti-Ms IgG (Vector Laboratories, Burlingame, CA) followed by streptavidin-horseradish peroxidase (Zymed Laboratories Inc.) and AEC substrate (Sigma, St. Louis, MO) as previously described.[14]

RESULTS

Monoclonal antibodies

One mAb against bottlenose dolphin peripheral blood mononuclear cell (PBMC) surface proteins was produced and characterized in the present study. This antibody was an immunoglobulin M (IgM) isotype.

Immunoprecipitation of target antigen

Immunoprecipitations were performed to determine the molecular weight of the antigen recognized by our mAb (Fig. 1). F21.H precipitated proteins of 180, 200 and 220×10^3 MW in different cell types (Fig. 1). Immunoprecipitation with bottlenose dolphin T cells resulted in a 180×10^3 MW band as well as a weaker band of 200×10^3 MW, while B cells precipitated a band of 220×10^3 MW as well as a weaker band of 200×10^3 MW. Immunoprecipitation of common dolphin

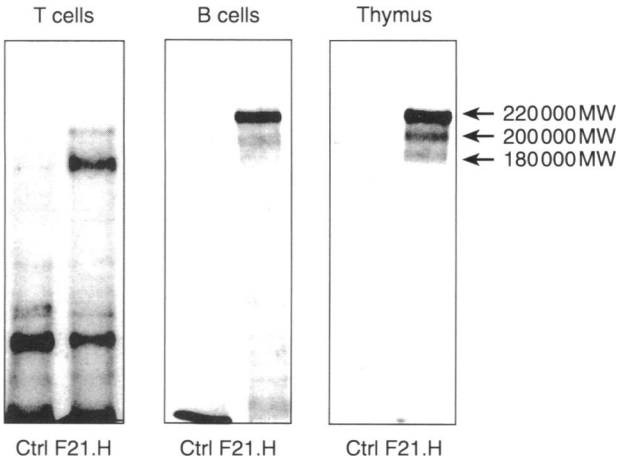

Figure 1. Immunoprecipitation of F21.H using bottlenose dolphin T lymphocytes and B lymphocytes, as well as common dolphin thymocytes. Each experiment included an irrelevant control antibody.

thymocytes resulted in a strong 220×10^3 MW band with an intermediate 200×10^3 MW band and a weaker 180×10^3 MW band.

Immunohistology

Sections of common dolphin lymph node were stained with F21.H to confirm its specificity for B or T-cell regions (Fig. 2). F21.H stained lymphocytes in both the follicles and the paracortex (Fig. 2a) of lymph node, and stained more intensely but not exclusively the medulla of thymus sections (Fig. 2b). Staining of thymus cortex was diffuse without unstained areas, but generally less intense than staining in the medulla.

Flow cytometric analysis

Relative percentages of PBMC from different species of cetaceans labelled with F21.H are shown in Table 1. F21.H cross-reacted with 34 to 75% of peripheral blood lymphocytes in all the species tested.

Representative histogram profiles of our mAb on single-cell suspensions of common dolphin thymus and lymph node are shown in Fig. 3. F21.H stained 79% of thymocytes and 86% of mesenteric lymph node cells.

Double labelling of bottlenose dolphin splenocytes was performed to assess the specific reactivity of our mAb. To study the distribution of the adhesion molecule recognized by F21.H, double labelling with either anti-CD21 or anti-CD2 was performed (Fig. 4). While 66% of the cells were labelled with both CD21 and F21.H, only 3% of the cells were labelled with anti-CD21 and not F21.H, and 10% were labelled with F21.H and not anti-CD21. Accordingly, 10% of the cells were labelled with both anti-CD2 and F21.H, 22% of the cells were labelled with anti-CD2 and not F21.H, and 60% were labelled with F21.H and not anti-CD2. Double labelling was also performed on killer whale peripheral blood lymphocytes using an anti-CD2 and F21.H antibody. The $CD2^+$ peripheral blood T lymphocytes that were negative for F21.H expressed a higher level of CD2 than did the $CD2^+$ cells positive for F21.H (Fig. 5).

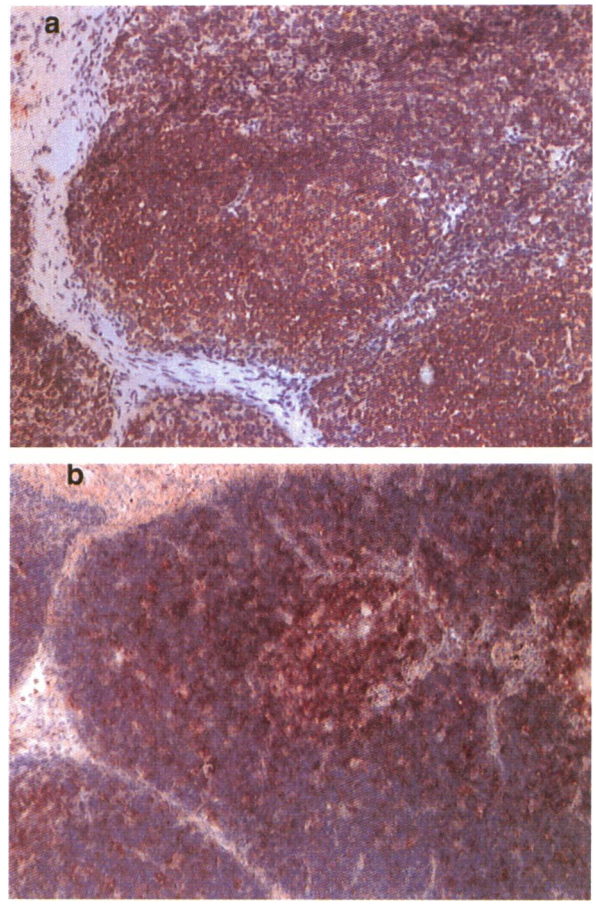

Figure 2. Sections of common dolphin (*Delphinus delphis*) lymph node (a) and thymus (b) stained with F21.H using immunoperoxidase. Sections were also stained with an irrelevant control antibody (not shown).

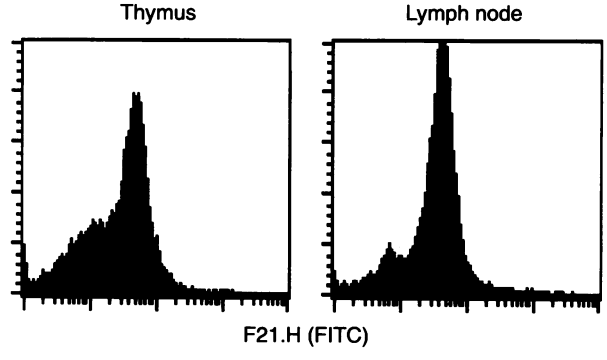

Figure 3. Representative histogram profiles of F21.H on single cell suspensions of common dolphin thymus and lymph node.

F21.H and T-cell activation

In order to study the effects of activation of cetacean T cells on their expression of the antigen recognized by F21.H, we evaluated the phenotype of killer whale peripheral blood lymphocytes, 0 to 4 days after concanavalin A (Con A) and phytohaemagglutinin (PHA) stimulation, using double labelling. The expression of CD45R at the surface of T lymphocytes was dramatically decreased upon stimulation by Con A and

Table 1. Relative percentages of peripheral blood mononuclear cells from different species of cetaceans labelled with F21.H

Species	%F21.H
Killer whale	67·30*
Orcinus orca	(16·68)†
	‡n=289
Bottlenose dolphin	43·59
Tursiops truncatus	(23·49)
	n=9
Beluga	43·36
Delphinapterus leucas	(8·04)
	n=14
Pacific white-sided dolphin	39·28
Lagenorhinchus obliquidens	(24·72)
	n=8
Striped dolphin	52·25
Stenella caeruleoalba	(16·36)
	n=6
False killer whale	71·15
Pseudorca crassidens	(9·55)
	n=4
Pilot whale	34·50
Globicephala melaena	(18·24)
	n=2
Bridled dolphin	47·60
Stenella frontalis	–
	n=1
Dall's porpoise	41·10
Phocoenoides dalli	–
	n=1
	n=1
Common dolphin	75·92
Delphinus delphis	–
	n=1

*Mean.
†Standard deviation.
‡n, number of samples.

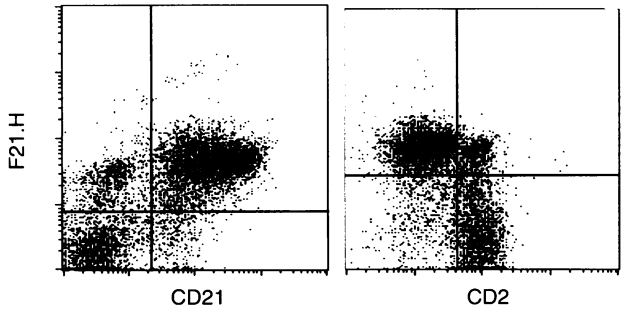

Figure 4. Double labelling of bottlenose dolphin splenocytes using monoclonal antibodies against cetacean B (CD21) and T (CD2) lymphocytes and F21.H.

PHA when compared with unstimulated control cells (Fig. 6). The decrease in CD45R expression was initially more rapid with PHA stimulation compared with Con A stimulation, and was relatively progressive versus time for the 4 days of the experiment.

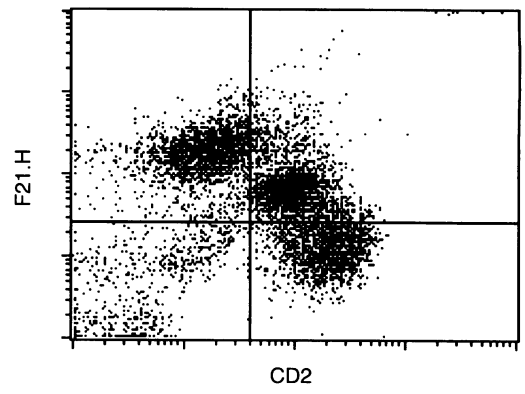

Figure 5. Double labelling of killer whale peripheral blood lymphocytes using monoclonal antibodies against cetacean T lymphocytes (CD2) and F21.H.

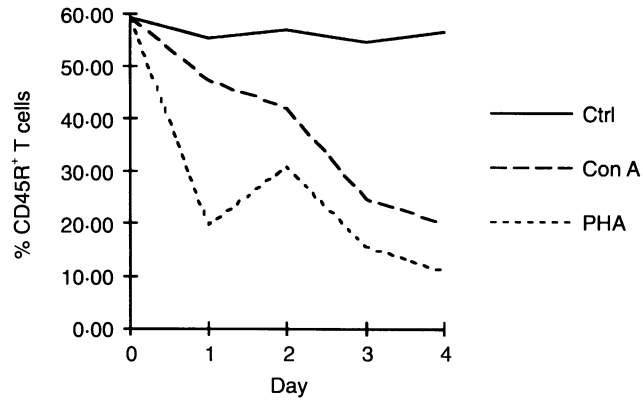

Figure 6. Loss of CD45R expression in killer whale (*Orcinus orca*) T lymphocytes upon stimulation with Con A and PHA. The each time point represents the average of three animals.

DISCUSSION

The present is the first report of the production and characterization of a mAb to a surface protein related to activation status of cetacean lymphocytes and similar to a CD antigen in humans and other species.

In order to confirm the specificity of our mAb, the molecular weight of the surface proteins it immunoprecipitated was determined. F21.H immunoprecipitated proteins of 180, 200 and 220×10^3 MW on various cell types. These proteins were of a similar size to the proteins precipitated by anti-CD45 (generally $180–220 \times 10^3$ MW) in a variety of species including human,[8] bovine,[4] ovine,[3] porcine,[16] feline,[17] guinea pig[18] and in *Xenopus*.[19] While some anti-CD45R mAb react against different forms of CD45 specific to different cell subtypes, others react against several forms of CD45 present on several cell subtypes.[20] Labelling of 39–71% of PBMC in different species of cetaceans by F21.H (Table 1) suggests that this antibody recognizes a restricted epitope of CD45 (CD45R). Those findings would be compatible with other groups of anti-CD45R that labelled 45–80% of PBMC in humans.[21] While immunoperoxidase showed labelling in both B and T-cell areas of lymph nodes (Fig. 2), double labelling demonstrated clearly that F21.H recognized isotypes of CD45 expressed on virtually all B lymphocytes but only on some T lymphocytes in

bottlenose dolphin splenocytes (Fig. 4). This pattern of staining in dolphin splenocytes corresponds with that of a group of anti-CD45R in rat that labelled all B cells and about 75% of T cells.[20] As anti-CD45RA, anti-CD45RB and anti-CD45RC are the only mAb that recognize isoforms larger than 200×10^3 MW, and only anti-CD45RB and anti-CD45RC react with epitopes on the $190–195 \times 10^3$ MW isoforms,[18] F21.H, which immunoprecipitates proteins of 180, 200 and 220×10^3 MW, is probably dependent on Exxon B or C. Furthermore, as about 80% of human thymocytes express the isoform recognized by anti-CD45RB[21] and only 2–3% of rat thymocytes express the isoform recognized by anti-CD45RC,[22] F21.H, which stained most thymocytes using immunoperoxidase and 79% of common dolphin thymocytes upon double labelling, would probably be best characterized, by analogy, as an anti-CD45RB.

F21.H immunoprecipitated proteins of different molecular weight in different cell subsets. The predominant expression of the high-molecular-weight form in B cells and multiple lower-molecular-weight forms in T cells is comparable with findings in other species.[7,21] The predominant expression of the high-molecular-weight form in common dolphin thymocytes seems to differ from most other species where the low-molecular-weight form (180×10^3 MW) is usually expressed.[7] This expression of the high-molecular-weight form of CD45R on thymocytes is not unique as anti-human PD7 as well as anti-feline 2F11 and 17B10, all mAb-recognizing molecular weights of 190, 205 and 220×10^3 MW, labelled 76%, 77% and 85% respectively of thymocytes.[17,21] A small amount of the 190×10^3 MW band was seen with the main 180×10^3 MW band upon immunoprecipitation of rat thymocytes with MRC OX-1.[20] As the expression of the high-molecular-weight form of CD45 appears to define the generative thymic lineage, as opposed to expression of the low-molecular-weight form of CD45 in cells committed to intrathymic death,[23] it could be hypothesized that this particular thymus was mainly composed of generative lineage, with most of the intrathymic selection having already taken place. This hypothesis would be supported by the older age (juvenile) of the dolphin sampled, and by the predominant labelling of thymocytes in the medulla (Fig. 2b), where the thymic generative lineage is known to be located.[24]

The expression of CD45R on killer whale T lymphocytes was reduced upon stimulation. This is similar to findings in humans where reactivity for different anti-CD45R monoclonal antibodies was reduced after PHA stimulation,[25,26] but different from cats where the expression of high-molecular-weight forms of CD45R was increased after Con A stimulation.[17] The loss of expression of CD45R in humans[25] was not as rapid as that observed in killer whales. It would be interesting to see if the loss of CD45R is associated with the development of memory in cetaceans as in humans.[25] It was also noted that killer whale T lymphocytes that were CD45R negative expressed a higher density of CD2 than CD45R-positive T cells, as did bovine memory T cells.[27]

The wide cross-reactivity of our monoclonal antibody between different species of cetaceans is not surprising as they all belong to the same family (*Delpninidae*), except for beluga whale (*Monodontidae*) and Dall's porpoise (*Phocoenidae*), and considering the close genetic relationship within the *Delpninidae*.[28]

There are potential diagnostic and therapeutic uses associated with the antibody we developed and characterized. A change in the isoform of CD45R present at the surface of T lymphocytes has been associated with their activation status,[25] and in some instances to their naive versus memory status.[25,29] In certain instances, such as in humans with human immunodeficiency virus (HIV), the selective impairment or loss of T-cell subsets defined by antibodies to isoforms of CD45R defines the clinical progression of the disease.[30] The recognition of activated killer whale T lymphocytes with F21.H makes it a promising tool for research and diagnostic applications, especially if loss of CD45R in T cells proves to be associated with memory.

ACKNOWLEDGMENTS

We would like to thank the veterinarians of Sea World Parks (James McBain, Tom Reidarson, Leslie Dalton, Mike Walsh, Sam Dover) for providing blood samples from different species of cetaceans as well as San Pedro Marine Mammal Center, Randy Wells (Mote Marine Laboratory) and Sam Ridgway (NOSC) for help collecting tissues from freshly dead cetaceans. SD is supported by a fellowship from the Medical Research Council of Canada.

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