Induction of Nitric Oxide Release by MRC OX-44 (anti-CD53) through a Protein Kinase C-dependent Pathway in Rat Macrophages

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Summary

Many membrane proteins are implicated in the control of cell function by triggering specific signaling pathways. There is a new family of membrane proteins, defined by its structural motifs, which includes several lymphoid antigens, but lacks a function. To study its biological role, we determined which signaling pathways are affected by the CD53 antigen, a prototypic member of this family, in rat macrophages. Activation of CD53 by cross-linking results in an increase in inositol phosphates and diacylglycerol and in Ca²⁺ mobilization, which are insensitive to pertussis or cholera toxins. There is a translocation of protein kinase C to the membrane accompanied by nitric oxide (NO) release in macrophages. This effect is the result of the expression of the inducible nitric oxide synthase (iNOS), which is dependent on protein kinase C and protein synthesis. These results have linked a new receptor with a specific pathway of NO induction and thus have opened up a novel aspect of NO regulation in cell biology.

A new lymphoid family of membrane proteins is characterized because its members are very hydrophobic and have four transmembrane domains; the NH₂ and COOH termini are intracytoplasmic, and they are glycosylated in one of the two extracellular domains (1). The family is known as the transmembrane 4 superfamily (TM4SF) proteins. Their structure is similar to that of connexins, the proteins forming gap junctions (2). These new TM4SF proteins have been related to the control of cell proliferation or to the tumor phenotype. Among the members of this family are CD37 (3), CD9 (4), the murine antigen TAPA1 which is related to the proliferative properties of T cells (5, 6), the antigen OX44/CD53 (7, 8), the human antigen ME491/CD63 which is a prognostic marker of melanoma (9, 10), the human antigen CO-029 originally detected in colon, rectal, gastric, and pancreatic carcinomas (11), the human R2 antigen present on most hematopoietic cells (12), and the human IA4 antigen (13). Because of the potential role they might play in the control of cell proliferation and differentiation, we tested the possibility that these proteins might be implicated in some signal transduction pathway and perhaps have a related biological property in all immune cells. To study this role, we chose the CD53 antigen because it is a panleukocyte antigen that is well characterized in rat and humans (8, 14–16).

The rat CD53 antigen is recognized by the mAb MRC OX-44 (7). This mAb defines a panleukocyte antigen molecule that is present in all mature cells of the immune system (1), including B and T lymphocytes, macrophages, monocytes, and leukocytes (7, 17). The presence of CD53 antigen on so many different cell types might represent a function common to all of them. In T cells CD53 defines a functional subpopulation during thymic development (7, 17) and enhances the cell response after stimulation of the TCR (17). In rat T cells, CD53 gene expression is induced by cross-linking of the T cell receptor (18) and CD53 coimmunoprecipitates with CD2 (15, 19) and modulates the associated tyrosine kinase activity (19) in T and NK cells. Recently, it has been shown that CD53 mediates signal transduction in human monocytes and B cells including an increase in cytosolic Ca²⁺ and the expression of characteristic responses such as the monocyte oxidative burst (20). Because in these studies CD53 was linked to important specific functions of monocytes, B or T cells, we reasoned that perhaps a similar linkage might occur in macrophages.

In the present work we show that CD53 is implicated in signal transduction in rat macrophages and induces the production of nitric oxide via a protein kinase C-dependent pathway. Nitric oxide plays a fundamental role in macrophage activation where it has both communication and defensive functions (21, 22).
Materials and Methods

Reagents and Chemicals. [U-14C]arginine, γ[32P]ATP and the kits for the assay of cGMP and inositol(1,4,5)trisphosphate (InsP3) from Amersham International (Amersham, UK). Adenosine 2',5'-bisphosphate–Sepharose was from Pharmacia LKB (Uppsala, Sweden). LPS was from Difco Laboratories, Inc. (Detroit, MI), and Dowex AG50W-X8 (Na+–form) was from Bio-Rad Laboratories (Richmond, CA). Nitrates reductase from Aspergillus niger and other enzymes were from Boehringer Mannheim (Mannheim, Germany) or Sigma Chemical Co. (St. Louis, MO). BH4 was from Dr. B. Schircks Laboratories (Jona, Switzerland).

mAbs. mAb MRC OX-44 (anti-CD53) and MRC OX-34 (anti-CD2) were obtained from Dr. A. Williams (Oxford University, UK) and have been previously described (7, 23). As a negative control we used monoclonal MARM (anti-rat IgM) from Serotec (Oxford, UK). The three mAbs are of the IgG1 isotype. For the preparation of F(ab')2 fragments, the mAb OX-44 was digested with pepsin and further purified by protein A-Sepharose chromatography following standard procedures (24).

Cell Cultures. Peritoneal macrophages were prepared from male rats and cells were cultured in RPMI 1640 medium supplemented with 10% of heat inactivated FCS (25). All cell culture material was from Gibco BRL (Gaithersburg, MD). Experiments were carried out in phenol-red–free DME medium supplemented with 1 mM arginine and 10% of heat inactivated FCS.

Determination of the Intracellular Ca2+ Concentration. Ca2+ concentration was measured in macrophages adhered to a coverslip (0.7 cm2) and loaded with 5 μM Fura 2-AM at 37°C for 20 min in the presence of 20 μg/ml of pluronic acid. After extensive washing, the coverslip was placed in a 1.5-ml spectrofluorometric cuvette containing PBS, 0.5 mM CaCl2, and 5 mg/ml of fatty acid–free BSA. The fluorescence was recorded at 510 nm in a spectrofluorometer (LS50; Perkin–Elmer Corp., Norwalk, CT) using a dual excitation source at 340 and 380 nm. The maximal fluorescence was determined at the end of the assay by adding 10 μl of 10% SDS. The minimal fluorescence was obtained by adding 15 μl of a solution containing 0.5 M EGTA, 0.5 M Tris, pH 9.0, and the spectra were analyzed after data export to Lotus 123 program (25). 2,5-Dit-(butyl)-1,4-benzohydroquinone (t-But-HQ) was used at 10 μM.

InsP3 and Diacylglycerol Determinations. The intracellular concentration of InsP3 was measured after extraction of the cell layer with 0.25 ml of ice-cold 0.5 M perchloric acid, and following the protocol of the kit supplier (Amersham International). To measure the 1,2-diacylglycerol (DAG) from 1,2-diacylglycerol (DAG) from 3 × 106 cells in 6-cm dishes, the cell layers were homogenized with 0.5 ml of ice-cold methanol, to which one volume of CH3OH/methanol (95:5) was added. After thorough mixing the tubes were centrifuged in a centrifuge (Eppendorf) for 10 min at 4°C and the organic layer collected. The DAG content was measured by its conversion to phosphatidic acid by diacylglycerol kinase from Esherichia coli, in the presence of γ[32P]ATP and using 1,2-diacylglycerol as internal standard (26).

Protein Kinase C Determinations. Macrophages (3 × 106 cells) were homogenized in 1 ml of 4 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 10 μg/ml leupeptin, and 20 mM Tris, pH 7.5. After centrifugation at 105,000 g for 30 min the activity present in the particulate fraction was extracted with homogenization medium supplemented with 0.1% NP-40. Both the soluble and the extracted fractions were purified by DE52 chromatography and the protein kinase C activity was assayed using histone H1 as substrate (26, 27). Western blot analysis of the particulate fraction was carried out using mAb specific for the α/β1/βII subspecies of protein kinase C (Seikagaku America, Inc., Rockville, MD).

CD53 and CD2 Analysis. To evaluate the macrophage population expressing CD53 and CD2 antigens, the cells were adhered to a coverslip and analyzed by immunofluorescence using as secondary antibody an FITC rat anti–mouse IgG1 antibody. At least 95% of the population resulted positive for the staining with OX-44, and under these conditions <5% of the cells were positive for the secondary antibody. In our system, we observed that virtually all the macrophage population was positive for the expression of CD53 (OX-44) and negative for CD2 (OX-34). The quantitation was performed after scraping cells from the slide and passing them through the FACScan (Becton Dickinson & Co., Mountain View, CA).

Determination of NO (Nitric Oxide). Total NO release was determined by the accumulation in the culture medium of nitrite and nitrate. Nitrites were reduced to nitrites with nitrates reductase, and nitrites were determined with Griess reagent by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration) (27). After a first reading of the absorbance at 595 nm, naphthylethylenediamine (1 mM in the assay) was added. The reaction was completed after 15 min of incubation and the absorbance at 595 nm was compared with a standard of NaNO2. The absorbance corresponding to samples stopped at time 0 of incubation was subtracted.

Cell Homogenates and Adenosine 2',5'-Bisphosphate-Sepharose Chromatography. Macrophage homogenates were prepared in 2 ml of a medium containing 20 mM Tris, pH 7.5 (4°C), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM 1,4-dithio-erythritol, 1 μM BH4, 1 μM leupeptin, and 0.2 mM phenylmethanesulfonyl fluoride (homogenization buffer). The cell homogenate was centrifugated at 20,000 g for 15 min, and the supernatant was partially purified by a 2',5'-ADP-Sepharose column (0.5 × 5 cm) equilibrated with homogenization buffer supplemented with 1 mM MgCl2 and 100 mM NaCl. After washing the column with this medium containing 0.5 M NaCl until no more protein emerged, nitric oxide synthase (NOS) activity was eluted in homogenized medium supplemented with 5 mM dithiocarbamid acid dinitriol phosphate (reduced) (NADPH) and 10% glycerol (vol/vol) (27). Fractions containing NOS activity were concentrated by ultrafiltration through a cellulose triacetate membrane with a cut-off of 30 kD (Sartorius, Gottingen, Germany). The presence of 1 μM BH4 during the purification was critical in maintaining an active enzyme. Enzyme assays were carried out immediately after purification. The protein concentration was measured in the pellet after ethanolic precipitation (28).

Assay of NOS Activity. The enzyme was assayed by the production of [U-14C]citrulline from [U-14C]arginine (27) in a buffer that contained 20 mM Hepes (pH 7.4), 30 μM [U-14C]arginine (0.3 μCi), 10 μM flavine adenine dinitriol, 10 μM BH4, and 0.5 mM NADPH (200 μl of incubation volume). After 10 min of incubation, the reaction was stopped with 1 ml of an ice-cold solution containing 10 mM EGTA, 1 mM citrulline, and 100 mM Pipes, pH 5.5. 1 ml of this mixture was applied to a 1 ml Dowex AG50W-X8 column (Na+–form), and [U-14C]citrulline was eluted in 3 ml of water. The reaction was carried out at 30°C and the enzyme activity was expressed as the difference of product formation in the absence or presence of 0.25 mM Nω-methyl-l-arginine in the reaction mixture. When the effect of Ca2+ on NOS activity was tested, the reaction mixture was incubated with 1 mM EGTA (basal activity) or in the presence of 10 μ/ml calmodulin and 100 μM Ca2+ (26, 27).
Northern Blot Analysis of NOS. RNA was extracted and analyzed by Northern blot using a 2.4-kb SspI/EcoRI probe isolated from a HincII-SspI fragment of NOS inserted into pUC19 (29). A Glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used to normalize the RNA load in gel lanes.

Determination of cGMP. To measure cGMP, the cells were incubated in 9-cm dishes for 10 min before ligand addition with 0.5 mM isobutyl-1-methylxanthine to prevent the degradation of cGMP. The medium was aspirated and replaced by 1 ml of an ice-cold mixture of ethanol/water (2:1, vol/vol). After homogenization and centrifugation in a centrifuge (Eppendorf, Inc.), samples were speed vacuum-dried and cGMP was measured using a specific binding kit, following the recommendation of the supplier (Amersham International).

Results

OX-44 and Its F(ab')2 Fragment Mobilize Ca2+ in Rat Macrophages. The identification of the early signals elicited after binding of extracellular ligands to membrane receptors is a useful approach to understand the pathways involved in the cellular response to the stimuli (30). To determine the signals generated by the cross-linking with mAb OX-44 or its F(ab')2 fragments in rat macrophages, we first measured the changes in the cytoplasmic Ca2+ levels. Cross-linking results in an immediate increase in cytosolic Ca2+ from the endoplasmic reticulum followed by a drop in its cytoplasmic level (Fig. 1 A). This suggests that CD53 cross-linking acts on Ca2+ mobilization at several levels. Similar results were obtained by using purified OX-44 F(ab')2 fragments (Fig. 1 B), suggesting that these effects are a consequence of antibody-mediated receptor cross-linking. No effect was detected when the Fc fragment or when another antibody of the same isotype as OX-44, IgG1, was used (data not shown). The response in Ca2+ mobilization was dependent on the concentration of OX-44 used (Fig. 1 C). Based on this response curve, we selected to use OX-44 at a concentration of 10 μg/ml unless otherwise indicated.

To confirm that the second phase of the response, the decrease in cytosolic Ca2+ (Fig. 1, A and B), was also a consequence of the cross-linking, we first released the endoplasmic reticulum pool with t-But-HQ, followed by the addition of mAb OX-44. In these conditions the mAb induces a reduction of the cytosolic Ca2+ (Fig. 1 D). The dual response that the antibody appears to exert points to an effect on the cell membrane, and this observation is in agreement with previous reports where complex effects on Ca2+ mobilization have been described (31). We further characterized the possible type of G protein implicated in this activation by studying the effects of different toxins, such as cholera and pertussis. In these experiments the toxins at a concentration of 1 μ/ml were added 15 min before the addition of OX-44. In agreement with recent results in monocytes and B cells (20), neither of the two toxins had an effect on mobilization of Ca2+ (Fig. 1 E and F), ruling out the involvement of Goα, and Gβγ proteins, but not of other G proteins in the transmembrane signaling through CD53.

OX-44 Activates the Production of InsP3 and DAG. The mobilization of intracellular Ca2+ suggested that it might be the consequence of a signal generated by phospholipase C activity (PLC). We therefore determined the level of the PLC reaction products, InsP3 and DAG, after cross-linking.
with OX-44. We examined the changes taking place during the 120 s after mAb addition to rat macrophages (Fig. 2). The levels of InsP3 increased fivefold immediately, a pattern of response typical of PLC activation. The levels of DAG also increased at least fivefold, but did not drop immediately (Fig. 2), suggesting the involvement of additional pathways, possibly a phospholipase D activity, in its generation (our manuscript in preparation). As Fig. 2 shows, macrophage treatment with an unrelated monoclonal antibody of the same isotype failed to elicit a response.

**Protein Kinase C Is Involved in the OX-44 Response.** The second messengers released after CD53 activation, like Ca2+ and DAG, are potential activators of protein kinase C. The activation of protein kinase C results in changes in its subcellular localization between soluble and membrane bound forms. To determine if OX-44 stimulator activates protein kinase C we measured the protein kinase C activity distribution between the soluble and particulate fractions in macrophages. As shown in Fig. 3, the addition of the antibody changes the distribution of protein kinase C towards its particulate form. This observation was further confirmed by Western blot analysis of the protein kinase C protein present in the particulate fraction (Fig. 3, insert) and was consistent with the redistribution of enzyme activity as a result of translocation (activation) after cross-linking with MRC OX-44 antibody.

**OX-44 Induces the Release of NO in Macrophages.** In macrophages NO has been reported to be an important effector molecule in response to several cytokines and endotoxins such as LPS (32), and a protein kinase C-dependent NO release has been observed in macrophages and hepatocytes (25, 27). Therefore, we determined the release of NO as a result of cross-linking of CD53 molecules with mAb MRC OX-44 in rat macrophages. As shown in Fig. 4, cross-linking with MRC OX-44 induces release of NO to an extent similar to that of LPS, a known activator of NO production in macrophages (33). Phorbol 12,13-dibutyrate (PDBu) is a phorbol ester that binds and activates protein kinase C, thus bypassing the need for an externally initiated signal, and it can be used to determine the full potential of the activation of this protein kinase C. The combination of LPS or PDBu with mAb OX-44 did not have an additive effect, nor completely activated the pathway. Inhibitors of protein kinase C, such as H7 and calphostin C, prevented the induction of NO release after CD53 cross-linking. These data suggest that the CD53 induction of NO release is mediated by protein kinase C and shares some common elements with the activation pathway triggered by LPS. The release of NO induced by CD53 cross-linking is also dependent on protein synthesis, as shown by its inhibition by actinomycin D or cycloheximide (Fig. 4).

**OX-44 Induces an Increase in the iNOS Activity.** NO is the reaction product of the NOS enzymatic activity (34). Recently, it has been shown that the activation of protein kinase C leads to the expression of the iNOS in macrophages (25), which is known to be induced by LPS or interferon-γ (33, 35). To study whether OX-44 could affect the induction of iNOS, we first performed the time course of this enzyme activity as a result of CD53 cross-linking. As shown in Fig. 5A there is an increase in NO activity after incubation with OX-44 that peaks at 4 h. This observation is consistent with the dependence on de novo protein synthesis for NO release previously shown by the use of protein synthesis inhibitors (Fig. 4). There are two major types of NOS enzymes (36); one is inducible and the other is constitutive, and they can be discriminated by the dependence of the con-

![Figure 3. Effect of mAb OX-44 on protein kinase C activity distribution between soluble (open bars, s) and particulate (dashed bars, p) fractions. The insert shows the distribution of protein kinase C of samples collected at 0, 2, 5, and 10 min after OX-44 addition and analyzed by Western blot. The blot was revealed using an anti-α/βI/βII protein kinase C antibody. Results are the means + SEM of three experiments.](image1)

![Figure 4. Effect of mAb OX-44 on the release of nitric oxide by rat macrophages incubated with protein kinase C modulators, endotoxins, and protein synthesis inhibitors. Macrophages were incubated for 6 h in the presence of the indicated ligands and the nitrite/nitrate release was measured with the Griess reagent. The nitrite content in unstimulated cells was 4.2 ± 0.3 nmol/mg of protein. Similar results were obtained when the macrophages were stimulated with OX-44 F(ab')2 fragment. Results are the means + SEM of three independent experiments.](image2)
Figure 5. Effects of OX-44 on NOS activity (A) and cGMP concentration (B). Macrophages were incubated with 10 μg/ml of MRC OX-44 (solid symbols) or the isotype control an anti-rat IgM (open symbols). NOS activity was measured at saturant concentrations of effectors and substrates, and the activity was independent of Ca²⁺ and calmodulin. Similar results to those of OX-44 were obtained with its F(ab)² fragment. Data are given as means + SEM of three independent experiments.

Discussion

CD53 is an ubiquitous antigen present on the membrane of all cells with a host defensive function (1). CD53 belongs to a membrane protein family, the TM4SF, which is highly conserved among its members and in different animal species, however its physiological role is very poorly understood (1, 16). In this work we have attempted to define a pathway in rat macrophages that could link this antigen to a known biological property of this cell type.

The immediate response to CD53 cross-linking, as shown by the effects of OX-44 or its F(ab)² fragment is the increase of intracellular Ca²⁺. This effect is complex and in addition to the mobilization of the intracellular stores, there is an additional target that results in the loss of the intracytoplasmic Ca²⁺, as shown by the effect of OX-44 in cells pretreated with t-But-HQ (Fig. 1 D). This Ca²⁺ mobilization in response to CD53 cross-linking is a common phenomenon to all cell types where it has been studied like monocytes, B, T, and NK cells, and consequently it might suggest a common transmembrane signaling in all these cells. Furthermore, this effect seems to be independent of the specific protein interactions that CD53 has on these cell types; thus in T cells it upregulates the effects of CD2 responses (15).

The activation of the InsP₃/DAG pathway has been linked to two types of membrane proteins (30). One with seven transmembrane domains, such as neuromodulin receptors (30), is mediated by a specific type of Gα proteins that can be dis-
ttinguished by the use of cholera and pertussis toxins, although other Gα proteins insensitive to both toxins (α6) have been reported (40). The other with one transmembrane domain, like platelet-derived growth factor and epidermal growth factor receptors, or complex receptors, like TCR, responds through tyrosine phosphorylation (30). However, the structure of these proteins is not related to any of the TM4SF type of proteins (16). Regarding CD53 it has been postulated that the Ox44/CD53 protein could form part of a channel for some small molecules (1), consistent with its structural homology to connexins (2). Because the proteins of the TM4SF family, including CD53, lack SH domains and are not known to be phosphorylated in tyrosine residues (14), they cannot be bound an SH domain (41). Therefore the transmission of its signal must be mediated through a novel connecting protein to protein kinases (42) that do not have a transmembrane domain (43). In the only TM4SF family member known to act through a tyrosine kinase mechanism, the TAPA-1 antigen in B cells, the connection is mediated through the CD19 molecule which has phosphorylated Tyr residues that can interact with SH2 domains (44). Tyrosine phosphorylation is an early event in the response to anti-TAPA-1 antibodies (44). Indeed, in human monocytes the Ca2+ mobilization induced by anti-CD53 antibodies seems to be affected by genistein, a nonspecific tyrosine kinase inhibitor (20). Therefore, the elucidation of the nature of the connecting molecules between the CD53 antigen and specific kinases constitutes a critical step in the understanding of the physiology of CD53 in each cell type.

Protein kinase C has been shown to be implicated in the induction of iNOS activity in rat hepatocytes treated with phorbol esters (26). In this work we have shown that protein kinase C activation after CD53 cross-linking triggers the expression of iNOS in rat macrophages. Thus, it is very likely that those agents that modulate iNOS expression might be functioning through a protein kinase C-dependent pathway.

The activation of the inducible form of iNOS by cross-linking of CD53 represents a novel mechanism by which this enzyme is regulated in macrophages where NO plays a key role in the response to infection and to tumor cells (21). This finding is important because CD53 is present in all mature types of the lymphoid lineage, including B and T cells, both in rats and humans. However, there must be differences in the function CD53 plays in each of these cell types. In rat T and NK cells, CD53 coprecipitates with CD2 and it enhances the response to the stimulation mediated by the T cell receptor (18, 19). In humans and rat, both macrophages and B cells are CD2 negative and lack TCR, thus CD53 interacts variously on the membrane and the functional differences are unknown regarding the specific aspects of the response to CD53 cross-linking among different cell types. Since the nature of the CD53 ligand is unknown, its identification will lead to a better understanding of NO biology. If other proteins of the same family such as CD53 also modulate the NO metabolism, new opportunities for research into this molecule and its role in cellular physiology will be opened. Understanding NO regulation will lead to the new knowledge about the modulation of cell communication in the immune system, and perhaps to a better management of clinical conditions such as hemorrhagic and septic shocks where NO is implicated (45). Nitric oxide besides its cytotoxic activity may have other functions in macrophages, like inducing vasodilation and tissue damage.

In this report we have demonstrated that in macrophages CD53 antigen cross-linking can induce the expression of iNOS and the release of NO through a protein kinase C-dependent pathway. Based on this work and in other reports on the effects of CD53 in monocytes, B and T cells, we can postulate that CD53 is a regulator of the specific functions of each cell type belonging to the immune systems.

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References

4. Boucheix, C., P. Benoit, P. Frachet, M. Billard, R.E. Wor-


