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Exocytosis of Arginine-Specific ADP-Ribosyltransferase and p33 Induced by A23187 and Calcium or Serum-Opsonized Zymosan in Chicken Polymorphonuclear Leukocytes[1]

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Exocytosis is a common phenomenon in neutrophil functions. We earlier reported the co-localization of arginine-specific ADP-ribosyltransferase [EC 2.4.2.31] and its target protein p33 (*mim-1* protein) in cytoplasmic granules in chicken polymorphonuclear leukocytes (so-called heterophils) [Mishima, K., Terashima, M., Obara, S., Yamada, K., Imai, K., and Shimoyama, M. (1991) *J. Biochem.* 110, 388-394]. In the present study, we obtained evidence that the transferase and p33 were released into the extracellular space by the stimulus of calcium ionophore A23187 or serum-opsonized zymosan, but scarcely by phorbol myristate acetate (PMA) or *N*-formyl-Met-Leu-Phe (fMLP), thereby indicating the co-localization of the transferase and p33 in the azurophilic granules, and not in specific granules. [^{32}P]ADP-ribosylation of p33 occurred in the extracellular space, induced by the stimulus of A23187 or opsonized zymosan in the presence of [^{32}P]NAD. Our findings are interpreted to mean that heterophil transferase and p33 may be involved in neutrophil functions during processes of inflammation.

Key words: ADP-ribosyltransferase, calcium, exocytosis, polymorphonuclear leukocytes, zymosan.

Mono-ADP-ribosylation is a post-translational modification in which the ADP-ribose moiety of NAD is transferred to various proteins and thus may be involved in the regulation of cellular processes.

Arginine-specific ADP-ribosyltransferases [EC 2.4.2.31] were identified in turkey erythrocytes (*1, 2*), rabbit skeletal muscle (*3-5*), and chicken peripheral polymorphonuclear leukocytes (so called heterophils) (*6*). The latter two transferases have been cloned (*5, 7*). Skeletal muscle transferase is located on the cell surface of skeletal muscle cells *via* the glycosylphosphatidylinositol (GPI) anchor and modifies integrin $\alpha 7$ (*8*). GPI-anchored arginine-specific ADP-ribosyltransferases detected on the cell surface of mouse cytotoxic T cells may suppress their proliferation (*9*). These enzymes were also detected in the chicken spleen membrane (*10*).

Among enzymes which use NAD as a substrate, cell surface antigen CD38 functions as an ADP-ribosyl cyclase and may be involved in calcium signaling and lymphocyte function (*11, 12*), and rat T cell antigen RT6 functions as an NAD glycohydrolase (*13*). These enzymes, including GPI-anchored ADP-ribosyltransferase, may function as ectoenzymes and are likely to regulate cellular responses to various stimuli.

We show here that chicken arginine-specific ADP-ribosyltransferase and its target protein p33, both present in cytoplasmic granules of peripheral heterophils (*6*), are released into the extracellular space by the stimulus of serum-opsonized zymosan or cation calcium ionophore A23187, but scarcely by phorbol myristate acetate (PMA) or *N*-formyl-Met-Leu-Phe (fMLP). Moreover, p33, a preferential substrate of the transferase (*6*), was ADP-ribosylated in the extracellular space. Evidence for this exocytosis leads to the notion that the chicken transferase could function as an exoenzyme and regulate cellular events such as attachment, migration, and phagocytosis during processes of inflammation.

MATERIALS AND METHODS

Materials—Chickens were obtained from a local slaughterhouse. [adenylate-^{32}P]NAD (29.6 TBq/mmol) were obtained from New England Nuclear. Ionophore A23187, Zymosan A, PMA, fMLP, and dry powder of *Micrococcus lysodeikticus* were purchased from Sigma Chemical. NAD and dithiothreitol were from Boehringer Mannheim.

Isolation of Peripheral Heterophils—Peripheral heterophils were isolated according to Mishima *et al.* (*6*). Briefly, the buffy coat of fresh chicken whole blood was layered on 12.5% Ficoll-Metrizoate solution [*X*% Ficoll-Metrizoate solution means a mixture of the *X*% concentration of Ficoll (Pharmacia) and 32.8% Metrizoate (Nycomed Pharma AS)

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Abbreviations: PMA, phorbol myristate acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GPI, glycosylphosphatidylinositol; HBSS, Hanks' balanced saline solution; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase.

in a ratio of 12:5] and centrifuged at 600×*g* for 15 min. The top layer was removed and washed with HBSS (Hanks' balanced saline solution), then layered on 4.5% Ficoll-Metrizoate solution, followed by centrifugation at 600×*g* for 15 min. Cells found in the bottom layer were mostly heterophils. To ensure the removal of erythrocytes, heterophil preparations were again subjected to 12.5% Ficoll-Metrizoate centrifugation and used for experiments after washing with HBSS.

Exocytosis Experiments—About 0.5–2×10^7 cells suspended in 1.0 ml of Ca^{2+},Mg^{2+}-free HBSS were incubated at 37°C for the indicated time in the presence or absence of stimuli at the indicated concentration. Incubation was terminated by rapid cooling in ice, and cell suspensions were centrifuged at 1,200×*g* for 2 min using a desk-top centrifuge. Cell-free supernatants were collected, and cell pellets were resuspended in 0.5 ml of Ca^{2+},Mg^{2+}-free HBSS with 0.1% Triton X-100, followed by ultrasonication. In some cases, cells treated with cytochalasin B (5 μg/ml) were preincubated for 10 min at 37°C before stimulation. In all exocytosis experiments, heterophils prepared from fresh blood samples were used. Opsonized zymosan was prepared as described by Kolotila and Diamond (*14*). Zymosan A was boiled in 0.9% saline for 30 min, washed twice in saline, suspended at 20 mg/ml and kept at 4°C. Before use, zymosan (5 mg/ml) was suspended in pooled chicken serum, opsonized for 30 min at 37°C on a rotatory shaker, washed twice in Ca^{2+}, Mg^{2+}-free HBSS followed by ultrasonication (20 s). The opsonized zymosan was stored on ice and used within the same day. A23187, fMLP, PMA, and cytochalasin B were all made up in dimethyl sulfoxide (DMSO) as described by Dewald and Baggiolini (*15*), and the final concentration of DMSO in the incubation mixture did not exceed 0.1% (v/v). One mM calcium used in some experiments caused maximum phagocytosis, as described by Absolom (*16*).

Enzyme and Protein Assays—For the standard assay of ADP-ribosyltransferase activity, the reaction mixture containing 20 μg of poly-L-arginine, 50 mM Tris/HCl (pH 9.0), 0.1 mM [adenylate-^{32}P]NAD (3.7 kBq/tube), 5 mM dithiothreitol, and 50 μl of the cell-free supernatant or 10 μl of the cell lysate in a total volume of 0.2 ml was incubated at 25°C for 15 min. The reaction was terminated by adding 10% trichloroacetic acid, and radioactivity in the acid-insoluble fraction was measured using a liquid scintillation spectrometer (Aloka, LSC-900).

β-Glucuronidase activity was measured fluorometrically with 4-methylumbelliferyl-β-D-glucuronide as substrate, as described by Dewald and Baggiolini (*15*).

Lysozyme activity was measured colorimetrically with *Micrococcus lysodeikticus* solution as substrate, as described by Jolle's (*17*).

The release of lactate dehydrogenase (LDH) served to estimate the cell membrane damage. LDH activity was determined by measuring the conversion of NADH to NAD during the conversion of pyruvate into lactate as described by Dewald *et al.* (*18*) and Absolom (*16*). The release of these enzymes was calculated as a percentage of total cellular content.

p33 was determined by SDS/PAGE followed by protein-staining or immunoblotting visualized with *p*-dimethylaminobenzaldehyde, using affinity-purified polyclonal antibodies generated by immunizing rabbits with purified p33, as described by Towbin *et al.* (*19*).

[^{32}*P*]*ADP-Ribosylation of Extracellularly Released Protein*—To demonstrate *in situ* ADP-ribosylation in the

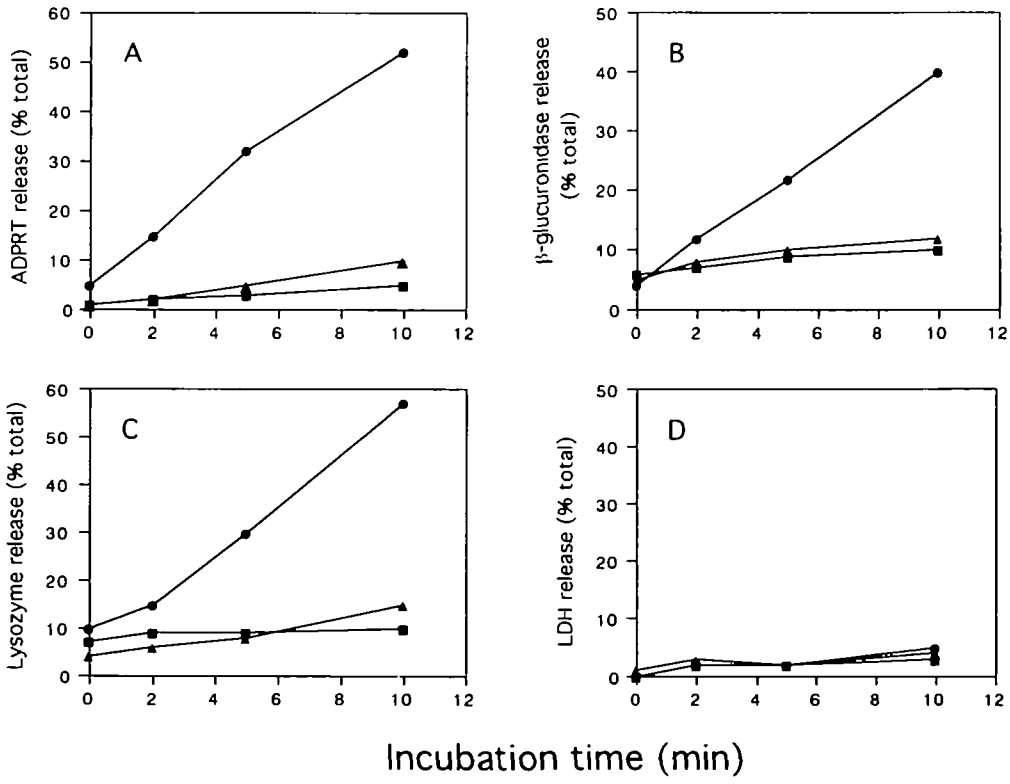

Fig. 1. **Effect of A23187 and/or calcium on the release of heterophil granule contents.** Heterophils were incubated with 5 μM A23187 (■), 1 mM calcium (▲), or both (●) for the indicated times at 37°C, and cell-free supernatants were collected. Activities of ADP-ribosyltransferase (A), β-glucuronidase (B), lysozyme (C), and LDH (D) in the supernatants were determined and expressed as percentage of the total activities found in nonincubated cells.

extracellular space, isolated heterophils were incubated with 50 μM [adenylate-^{32}P]NAD (55 kBq/tube) and 5 μM A23187 and/or 1 mM $CaCl_2$, or 2 mg/ml zymosan or opsonized zymosan at 37°C, for the indicated time. After incubation, the supernatants were removed by centrifugation at 1,200 × g for 2 min. The acid-insoluble fractions of the cell-free supernatants were analyzed by 15% SDS/PAGE followed by autoradiography.

SDS/PAGE—SDS/PAGE was performed according to Laemmli (*20*). Gels were stained with Coomassie Brilliant

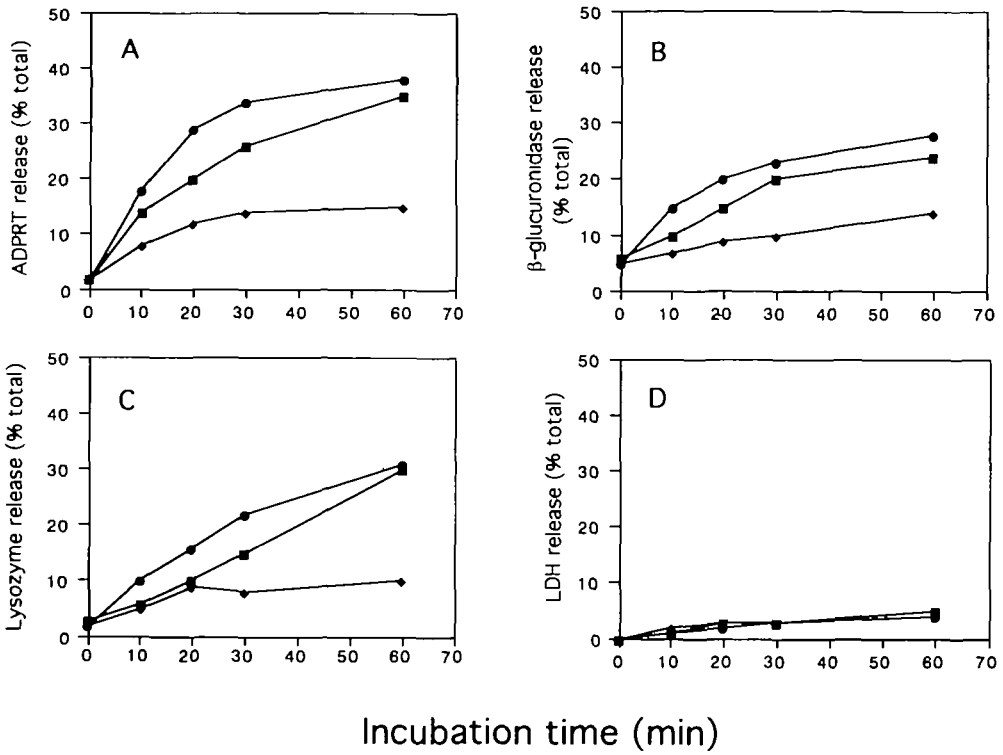

Fig. 2. **Effect of zymosans on the release of heterophil granule contents.** Heterophils pretreated with (●) or without (◆, ■) cytochalasin B were incubated further with 2 mg/ml zymosan (◆) or 2 mg/ml opsonized zymosan (■, ●) for the indicated times, and enzyme activities in cell-free supernatants obtained after these treatments were measured. Each panel shows enzyme release as described in the legend to Fig. 1.

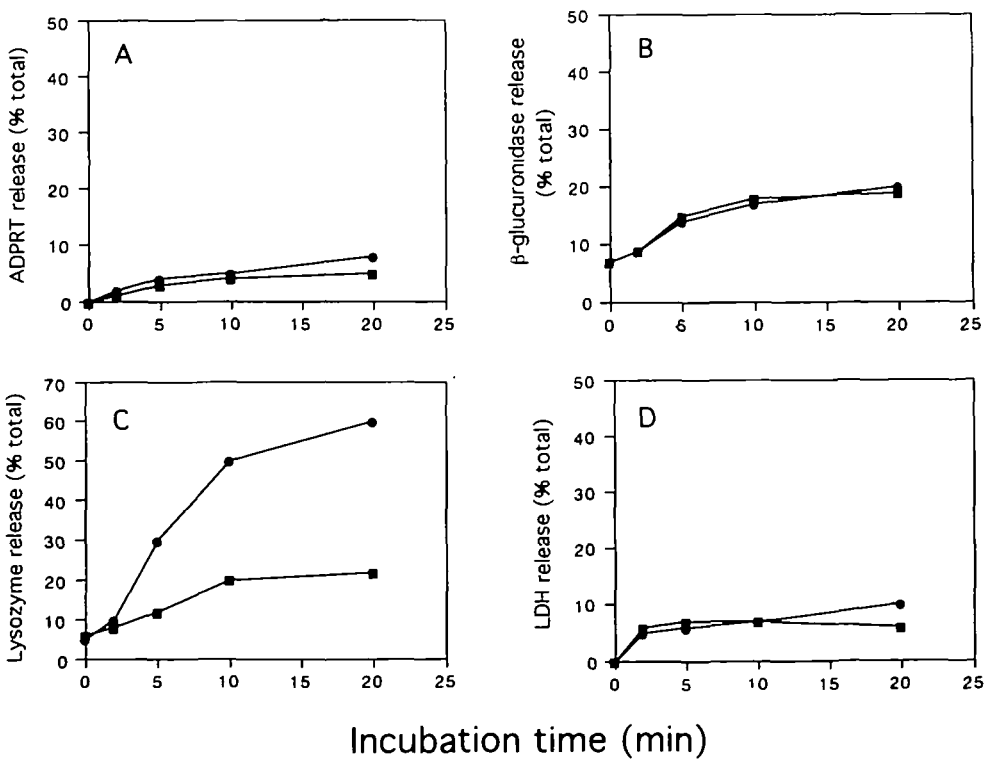

Fig. 3. **Effect of PMA and DMSO on the release of the heterophil granule contents.** Heterophils were incubated with 20 ng/ml PMA (●) or 0.1% DMSO (■) for the indicated time and enzyme activities in cell-free supernatants obtained after incubation were determined. Each panel shows enzyme release as described in the legend to Fig. 1.

Blue G-250 and autoradiographed on Kodak Tmat-G films for the appropriate time. Molecular markers used were purchased from Pharmacia; the molecular mass is given for each figure.

RESULTS

Release from Heterophils of Arginine-Specific ADP-Ribosyltransferase and Marker Enzymes—We found in earlier work that arginine-specific ADP-ribosyltransferase and p33 were co-localized in secretory granule fractions of chicken heterophils (*6*). Here we investigated whether the enzyme or protein could be released from the cells by stimuli such as calcium ionophore A23187, opsonized zymosan, PMA, or fMLP.

Peripheral heterophils were incubated with 5 μM calcium ionophore A23187 and/or 1 mM calcium at 37°C for the indicated time and cell-free supernatants were assayed for ADP-ribosyltransferase and three marker enzymes: β-glucuronidase (a representative of azurophilic acid hydrolases), lysozyme (a representative of both azurophilic and specific granule enzymes), and LDH (a marker enzyme of cytosol). As depicted in Fig. 1A, ADP-ribosyltransferase activity in the cell-free supernatant increased with increasing incubation time with 5 μM A23187 plus 1 mM calcium, and the transferase activity reached 50% of the total content at 10 min. No or little increase was observed with 5 μM A23187 or 1 mM calcium only. Prolonged incubation with 5 μM A23187 and 1 mM calcium did not further increase the transferase activity. Simultaneously, β-glucuronidase and lysozyme were secreted in a similar fashion (Fig. 1, B and C), but LDH release did not increase during 10 min of incubation with stimulants (Fig. 1D). The activities of ADP-ribosyltransferase and three marker enzymes in the cell pellets decreased in proportion to increase in the activities in cell-free supernatants (data not shown). These results indicate that the increase in the ADP-ribosyltransferase activity in cell-free supernatants was related to secretion of granule contents and not to rupture of the cell membrane. Trypan blue dye exclusion tests also confirmed the cell viability in these experiments (data not shown).

With opsonized zymosan, cytochalasin B-treated heterophils released larger amounts of the transferase, β-glucuronidase, and lysozyme than did untreated cells; release of these enzymes showed a similar percentage at 60 min (Fig. 2). A longer time was required to reach the maximum release of these enzymes with zymosan than with A23187; particulate stimulants such as zymosan tend to activate neutrophils, and they degranulate more slowly than do soluble stimulants (*21*). This opsonized zymosan is representative of phagocytable particles, which are ingested by the phagocytic white blood cells. These data suggest that release of ADP-ribosyltransferase during phagocytosis is feasible.

PMA did not enhance the release of the transferase or β-glucuronidase compared with the former two stimulants (Fig. 3). Only lysozyme was released by stimulation with PMA. We also examined the effect of 0.1-1.0 μM fMLP on the release of the enzymes from heterophils and the results were much the same as in the case of PMA (data not shown).

Release of p33—We investigated the release of p33 using the same methods as for the release of transferase and marker enzymes. Figure 4, A and B, shows respectively the effect of the calcium ionophore A23187 and serum-opsonized zymosan on the release of p33 from the heterophils. p33 was also identified by immunoblotting using affinity-purified polyclonal antibodies against p33 (Fig. 4C). These experiments demonstrate that, like the transferase, p33 was indeed released by calcium ionophore A23187 and opsonized zymosan, but not by PMA or fMLP. The manner of release of ADP-ribosyltransferase and p33 was quite similar, consistent with their co-localization in granules (*6*). It was noteworthy that p33 was a major protein released from the heterophils by the stimuli (Fig. 4, A and B).

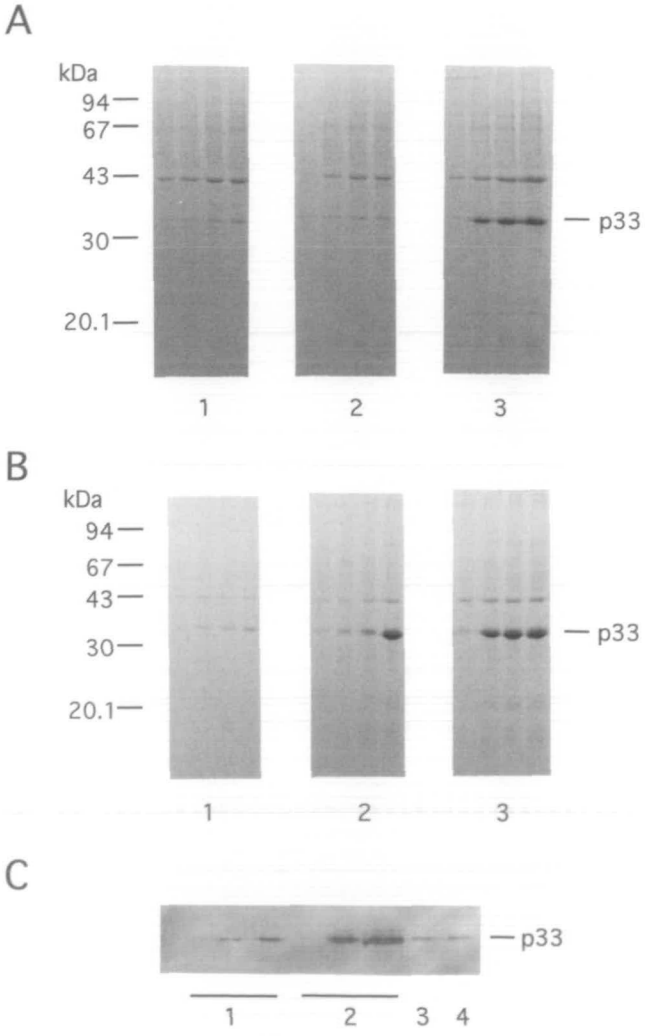

Fig. 4. **Time course of p33 release induced by 5 μM A23187 and 2 mg/ml opsonized zymosan.** Heterophils were incubated at 37°C with 1 mM calcium (1), 5 μM A23187 (2) or both (3) for 0, 2, 5, and 10 min in [A]. In [B], heterophils pretreated with (3) or without (1 and 2) cytochalasin B were incubated with 2 mg/ml zymosan (1), 2 mg/ml opsonized zymosan (2 and 3) at 37°C for 0, 15, 30, and 60 min. After incubation, cell-free supernatants were analyzed by SDS/PAGE and stained with Coomassie Brilliant Blue ([A] and [B]). In [C], heterophils were incubated at 37°C with 2 mg/ml zymosan (1) or 2 mg/ml opsonized zymosan (2) for 0, 10, and 30 min, with 1 μM fMLP for 10 min (3), or with 20 ng/ml PMA for 20 min (4). After incubation, p33 released into cell-free supernatant was detected by Western blot analysis with anti-p33 antibodies.

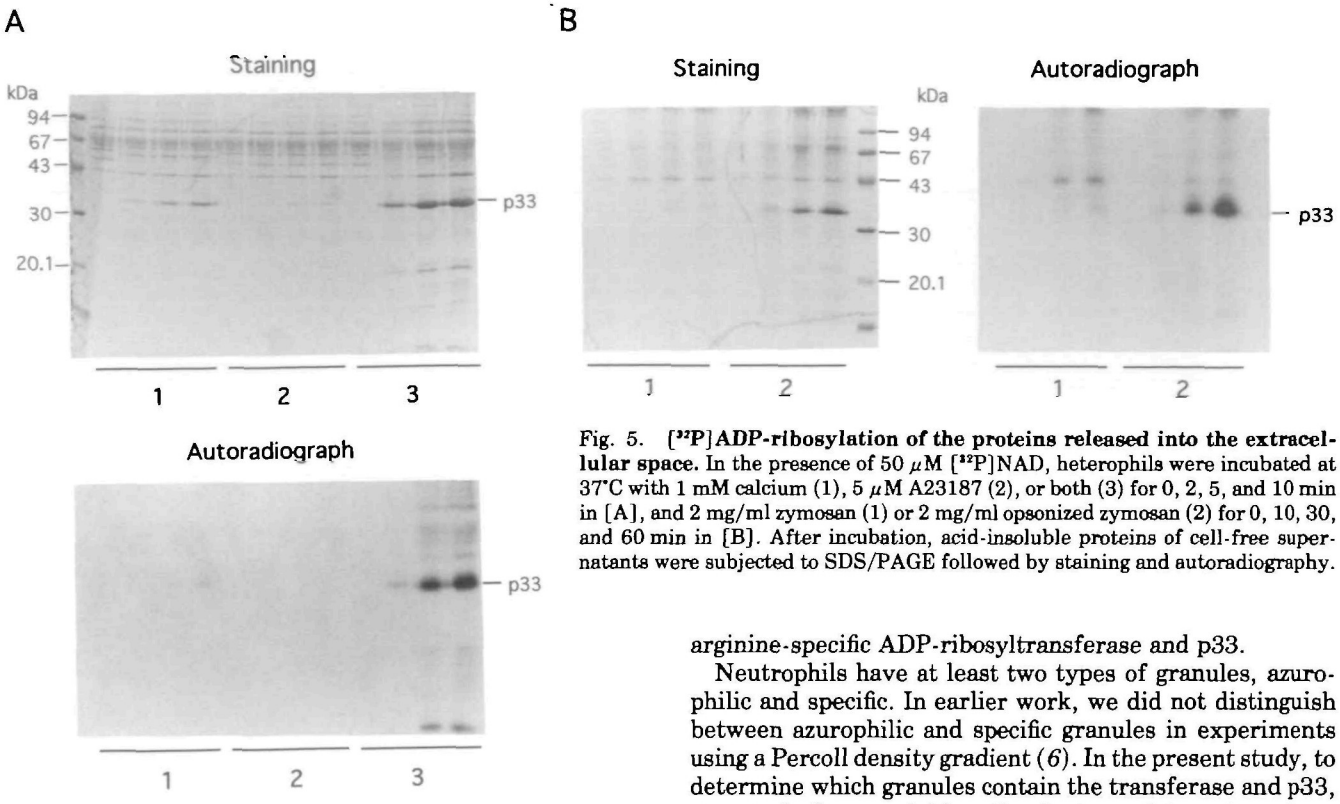

Fig. 5. **[^{32}P]ADP-ribosylation of the proteins released into the extracellular space.** In the presence of 50 μM [^{32}P]NAD, heterophils were incubated at 37°C with 1 mM calcium (1), 5 μM A23187 (2), or both (3) for 0, 2, 5, and 10 min in [A], and 2 mg/ml zymosan (1) or 2 mg/ml opsonized zymosan (2) for 0, 10, 30, and 60 min in [B]. After incubation, acid-insoluble proteins of cell-free supernatants were subjected to SDS/PAGE followed by staining and autoradiography.

[^{32}P]ADP-Ribosylation of p33 Released into the Extracellular Space—We reported that p33 was a preferential substrate for chicken ADP-ribosyltransferase *in vitro* and *in situ* (*6*). Since both proteins were confirmed to be released from the granules (Fig. 4), we asked whether p33 would be modified by the transferase in the extracellular space, in response to stimulation. We added 50 μM [adenylate-^{32}P]NAD to the suspension of heterophils and stimulated the cells with 5 μM calcium ionophore A23187 or 2 mg/ml opsonized zymosan. As shown in Fig. 5, p33 was ADP-ribosylated by either stimulant. p33 was also modified to a similar extent when [^{32}P]NAD was added into the cell-free supernatants of the heterophils induced by either stimulant (data not shown). These results indicate that, in the extracellular space, p33 was modified by the concomitantly released ADP-ribosyltransferase in the presence of NAD.

DISCUSSION

Neutrophils exercise their functions in primary host defense against pathogens through different effector systems, including superoxide generation and granule secretion. Cytoplasmic granules of neutrophils play a role mainly in microbial killing and possess a variety of lysozomal enzymes. These contents of the granules are released during phagocytosis, whereby they are secreted into phagocytic vesicles, or by secretion from the cells, so that they may act extracellularly (*21*). We found that arginine-specific ADP-ribosyltransferase and p33 co-localized in chicken heterophil granules can be released by calcium ionophore A23187 or opsonized-zymosan, but not by PMA or fMLP alone. This seems to be the first evidence of exocytosis of eukaryotic arginine-specific ADP-ribosyltransferase and p33.

Neutrophils have at least two types of granules, azurophilic and specific. In earlier work, we did not distinguish between azurophilic and specific granules in experiments using a Percoll density gradient (*6*). In the present study, to determine which granules contain the transferase and p33, we used three soluble stimulants, calcium ionophore A23187, PMA, and fMLP, to induce exocytosis of the transferase and p33. In neutrophils, intracellular calcium transients are crucial signal transduction events that mediate granule exocytosis (*22-24*). Calcium ionophore can induce the transport of calcium across the membrane, and ionophore-induced exocytosis depends on the presence of calcium in the extracellular spapce (*23*). Low concentrations of calcium ionophore A23187 (0.01-1.0 μM) lead to discharge of specific granules from neutrophils in the presence of extracellular calcium, while higher concentrations of the ionophore (10-20 μM) cause exocytosis of both azurophilic and secondary granules (*25-28*). We also tested A23187 at concentrations of 0.1-1.0 and 10 μM, and observed that 0.1-1.0 μM A23187 elicited up to 10% release (percentage of total) of ADP-ribosyltransferase and p33, while 10 μM A23187 led to 70% release during 10 min of incubated at 37°C (data not shown). These data suggest that the transferase and p33 are co-localized in azurophilic granules rather than in specific granules. PMA induces the selective degranulation of specific granules (*29-31*). The potent chemotactic peptide fMLP also degranulates specific granules, and only low levels of secretion of azurophilic granules are observed upon stimulation of neutrophils with higher concentrations (*e.g.*, 1 μM) (*21*). In the absence of cytochalasin B, elevated degranulation of ADP-ribosyltransferase and p33 was not induced by 20 ng/ml PMA or 0.1-1.0 μM fMLP.

Human neutrophils have two additional and distinguishable granules, gelatinase-containing ones and secretory vesicles (*32, 33*), but whether these are present in the chicken is not clear. These granules are more sensitive to cytosolic calcium than are azurophilic granules (*24*). We found that degranulation of the transferase and p33 was less sensitive to a low concentration of A23187 or calcium

(data not shown), thereby ruling out the existence of the transferase and p33 in these granules.

While endogenous ADP-ribosyltransferases have been detected in various species and tissues, their precise functions are not well understood. Chicken arginine-specific ADP-ribosyltransferase has 50% homology with the deduced amino acid sequence for rabbit skeletal muscle cell transferase and 35% homology with rat T cell alloantigens RT6.1 and RT6.2, which show NAD glycohydrolase activity (*7, 13*). The analysis of chicken ADP-ribosyltransferase cDNA indicated that the transferase has a leader or signal peptide in the N-terminus, suggesting that it is secretable (*7*). Skeletal muscle transferase and RT6 exist on cell surface, GPI-anchored to the membrane, and may function as ectoenzymes through metabolism of NAD or the ADP-ribose moiety (*5, 8, 13*). The heterophil transferase is located in secretary granules and could be excreted into the extracellular space by secretagogues. This transferase actively functions as an exoenzyme in the extracellular space, rather than as an ectoenzyme. p33, a preferential substrate of the transferase *in vitro* and *in situ*, was also ADP-ribosylated in the extracellular space in the presence of NAD (see Fig. 5). The amount of NAD in normal serum was scanty. For example, NAD concentrations in white or red blood cells and normal serum, detected by HPLC, were calculated to be 100–150 μM and a few nM, respectively, in the case of chicken (Terashima, M., unpublished observations). Damaged or dead cells may supply NAD in special milieus such as sites of inflammation.

p33 is an abundant protein in the granules of heterophils (*6*) and was identified as product of the *mim-1* gene, the expression of which is regulated by an oncogene product, Myb (*34*). High levels of expression and storage in promyelocyte granules indicate that p33 (*mim-1* protein) might play an important role as a structural component of the granules or secreted protein (*35*). Our present observations shed light on the physiological role of p33.

Neutrophil functions during inflammation involve margination, diapedesis, migration upward to the chemoattractant gradient, phagocytosis, and degranulation (*21*). In the present study, we obtained evidence that arginine-specific ADP-ribosyltransferase and p33 (*mim-1* protein) were released from chicken heterophils. This feature of the transferase and p33 provides clues to resolve the physiological role of these proteins in numerous functions of neutrophils.

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