Vol. 152, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1988 Pages 136-142

NEUTROPHIL ACTIVATION BY SURFACE BOUND IgG: PERTUSSIS TOXIN INSENSITIVE ACTIVATION

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Received February 19, 1988

SUMMARY: Surface bound IgG induces neutrophil degranulation and production of superoxide radicals by a mechanism that is not inhibited by either pertussis toxin or cholera toxin, whereas these functions induced by soluble mediators such as FMLP and soluble aggregates of IgG are profoundly inhibited by pertussis toxin. Interaction of neutrophils with surface bound IgG triggers the loss of ^{32}P labeled PIP_2 and PIP and the influx of extracellular calcium. Neither of these cellular events when induced by surface bound IgG is inhibited by pertussis toxin. These observations suggest that neutrophil activation induced by surface bound IgG proceeds along a pathway which is not regulated by proteins which are inhibited by either pertussis or cholera toxins.

The secretory products of activated neutrophils including neutral proteases and reactive oxygen metabolites contribute to the tissue injury in a number of inflammatory disorders (1). These neutrophil products are released after a specific ligand interacts with a membrane receptor inducing an activation signal. Soluble ligands such as the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) trigger a series of events which include the activation of phospholipase C leading to the generation of the second messengers inositol tris-phosphate and diacylglycerol (2-4). Activation of phospholipase C is dependent upon a guanine nucleotide regulatory protein (G protein) (5,6). This G protein can be inactivated by ribosylation in the presence of pertussis toxin (7). Pertussis toxin, presumably by inactivating this G protein, blocks the FMLP induced metabolism of membrane

0006-291X/88 $1.50

polyphosphoinositides, elevation of intracellular calcium, the production of oxygen radicals, and degranulation (8-12).

Another mechanism of activation occurs when neutrophils engage a tissue bound immune complex. The interaction of this ligand with the Fc receptor on the plasma membrane does not result in internalization of the ligand. Neutrophil activation by this mechanism is of clear pathophysiologic importance, since proteases released in this manner are more likely to encounter contiguous, degradable tissue molecules prior to inactivation by protease inhibitors. The activation signal triggered by interaction of the neutrophil with surface bound immunoglobulins is the object of this study.

MATERIALS AND METHODS

Materials: Cohn fraction II, FMLP, ferric cytochrome C, lanthanum chloride and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis Mo. Human IgG was purified from Cohn fraction II as previously described (13). Soluble aggregates of IgG were formed by heating purified IgG to 60°C followed by sodium sulfate precipitation (13). Pertussis toxin was purchased from List Biochemicals, Campbell, Ca. ^{32}P-inorganic phosphate (8mCi/ml) and $^{45}Ca^{++}$ were purchased from Amersham Corp., Arlington Heights, Ill.

Methods: Blood was collected from healthy adult volunteers and neutrophils were isolated by established procedures (14). For functional assays neutrophils (1.25×10^6 cells /ml) were incubated (30 min,37°) in 96 well microtiter plates coated with IgG or in BSA coated wells containing either aggregated IgG or FMLP. Upon completion of the incubation period, the wells were aspirated; the aspirates were individually centrifuged (300Xg,10 min), and the cell free supernatants assayed for lactoferrin, cathepsin G, and elastase by radioimmunoassay as a measure of degranulation (14). Superoxide production was measured spectrophotometrically by following the reduction of ferric cytochrome C (15).

Calcium influx was determined by measuring the influx of $^{45}Ca^{++}$ (16). Neutrophils, $3x10^6$ cells/ml in buffer containing 10^6 cpm of $^{45}Ca^{++}$/ml were placed in tubes coated with either IgG or with BSA. After incubation (5 min, 37°C) tubes were aspirated and aspirates were placed over Millipore filters. Tubes and filters were washed with five volumes of isotonic buffer containing 1mM EGTA. Filters were dried and counted for radioactivity.

To quantitate phosphatidylinositol bis 4,5 phosphate,(PIP_2) and phosphatidylinositol phosphate (PIP), neutrophils were labeled with ^{32}P as described by White et al. (17). Neutrophils (10^8 cells/ml) dispersed in phosphate free buffer (17) were incubated (90 min, 37°C) in the presence of ^{32}P-inorganic phosphate (0.5 mCi/ml). The ^{32}P-labelled neutrophils were then washed twice in phosphate free buffer, resuspended at 3×10^7 cells/ml and added to glass tubes coated with either BSA or IgG. Reactions were terminated by the addition of chloroform: methanol:HCl (100:200:2 v/v/v). Lipids extracts were prepared as previously described (18,19) and phospholipids were separated by thin layer chromotography. After autoradiography, phospholipids were identified by comparison to authentic standards. Identified spots were scraped into scintillation vials and counted for radioactivity.

RESULTS

Degranulation in response to either FMLP or soluble aggregates of IgG was, as previously reported (20), blocked by pre-incubation with pertussis

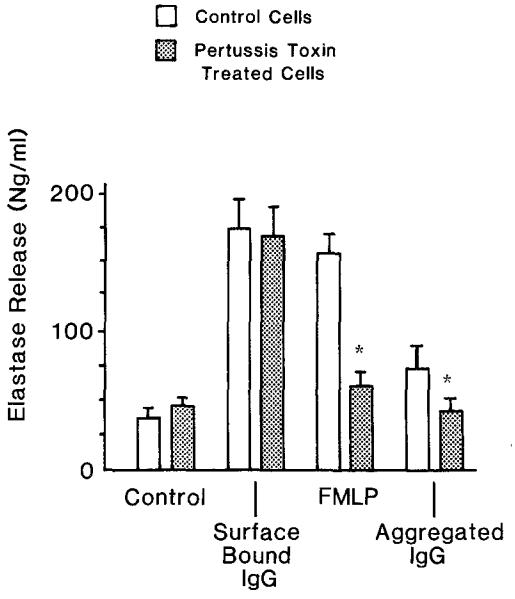
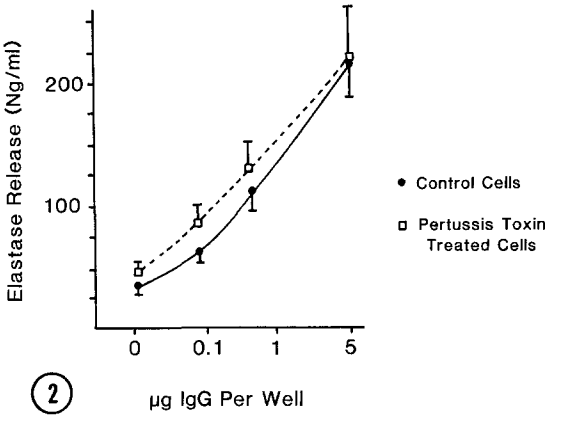


Figure 1: Effect of pertussis toxin on neutrophil degranulation. Neutrophils (1.25×10^6/ml) were incubated with or without pertussis toxin (2000 ng/ml). The cells were aliquoted (250 ul) into wells of microtiter plates which had been precoated with either IgG (5ug) or BSA (1%). Into some BSA coated wells FMLP (10^{-6}M) or aggregated IgG (200 ug/ml) was added. Degranulation was measured by assaying the release of elastase as described in "Materials and Methods". Each point is the mean of six different determinations; the standard deviation is indicated by the bars. * indicates elastase release by pertussis toxin treated cells is different from control cells at <0.01 significance level.

toxin (figure 1). In contrast, degranulation in response to surface bound IgG was not inhibited by pre-incubation with pertussis toxin. Pre-incubation with pertussis toxin in concentrations ranging from 50 to 4000 ng/ml or for times ranging from 90 to 300 minutes had no effect on degranulation mediated by surface bound IgG. Neutrophils incubated in wells which had been coated with lesser amounts of IgG, released lesser amounts of granule proteins. Pre-incubation of neutrophils with pertussis toxin did not alter the amount of granule proteins released into IgG coated wells, regardless of the amount of IgG used to coat the wells (figure 2).

Production of superoxide radicals by neutrophils in response to surface bound IgG was also not inhibited by pre-incubation with pertussis toxin, whereas the same response to either FMLP or soluble aggregates of IgG was strongly inhibited by pertussis toxin (figure 3). The concentration of pertussis toxin used or the time of exposure to pertussis toxin did not affect the generation of superoxide radicals induced by surface bound IgG.

Incubation with cholera toxin had no effect on the release of granule proteins or production of superoxide radicals by neutrophils stimulated by surface bound IgG, soluble aggregates of IgG, or FMLP (data not shown).



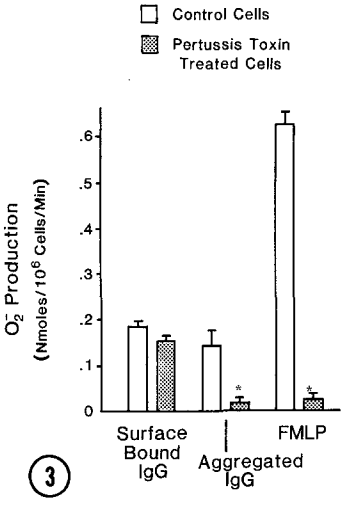


Figure 2: Effect of pertussis toxin on surface bound IgG mediated degranulation. Neutrophils (1.25 X 10^6/ml) were incubated (120 min, 37°C) with or without pertussis toxin (2000 ng/ml). The cells were aliquoted (250 ul) into wells of microtiter plates which had been precoated with the indicated amounts of human IgG. Elastase release was measured as described in "Materials and Methods". Each point is the mean of four different determinations; the standard deviation is indicated by the bars.

Figure 3: Effect of pertussis toxin on neutrophil superoxide production. Neutrophils (1.25 X 10^6/ml) were incubated (120 min, 37°C) with or without pertussis toxin (2000 ng/ml). The cells were aliquoted (190 ul) into wells of microtiter plates which had been precoated with either human IgG (5ug) or BSA. In some BSA coated wells FMLP (10^{-6}M) or aggregated IgG (200ug/ml) was added. Superoxide production was measured spectrophotometrically as described in "Materials and Methods." Each point is the mean of six different determinations; the standard deviation is indicated by the bars. * indicates that superoxide production by pertussis toxin treated cells is different from control cells at <0.01 significance level.

Table 1 demonstrates that there is loss of ^{32}P-PIP$_2$ and to a lesser extent ^{32}P-PIP from intact neutrophils which were incubated in glass tubes coated with IgG. Preincubation of neutrophils with pertussis toxin did not inhibit this loss of labelled PIP$_2$ and PIP.

Influx of calcium, as measured by $^{45}Ca^{++}$ uptake was induced by incubating neutrophils in wells containing surface bound IgG. The amount of calcium incorporation was slightly less than than incorporated by neutrophils incubated with 10^{-6}M FMLP (Table 2). Pertussis toxin did not inhibit the enhanced influx of ^{45}Ca triggered by surface bound IgG (data not shown). To further evaluate the physiologic importance of calcium influx in neutrophil activation by solid phase IgG, calcium influx was blocked with the use of lanthanum chloride (21,22). However, release of granule proteins extracellularly (Table 2) and the production of superoxide radicals (data not shown) was only slightly inhibited by incubation with lanthanum chloride. Enhanced calcium influx, however, as measured by uptake of $^{45}Ca^{++}$ was effectively blocked by pretreatment with lanthanum chloride.

Table 1. Lack of effect of pertussis toxin on surface bound IgG mediated loss of PIP_2 and PIP

	PIP_2		PIP	
PERTUSSIS TOXIN	-	+	-	+
CONTROL	100±5	100±2	100±3	93±3
SURFACE BOUND IgG	86±6*	86±3*	88±3*	84±8*

Human neutrophils which had been labeled with ^{32}P as described in "Materials and Methods" and incubated (37°C, 120 min) with or without pertussis toxin (2000ng/ml) were incubated (30 seconds) in glass tubes precoated with either human IgG or BSA. PIP_2 and PIP were extracted and quantitated as described in "Materials and Methods". Each point represents the mean and standard deviation of six determinations expressed as percentage of control. * Indicates a $p<0.025$ when compared to control.

DISCUSSION

Neutrophils are activated by engagement of their Fc receptors by both soluble aggregates of IgG and surface bound immunoglobulins. Previous studies (20) and our results indicate that soluble aggregates of IgG, like FMLP, induce activation of the neutrophil by a mechanism which is inhibited by pertussis toxin. In contrast, surface bound IgG activates neutrophils by a mechanism not sensitive to inhibition by pertussis toxin. Neutrophil activation by surface bound IgG, like activation by FMLP, is also not inhibited by cholera toxin.

Metabolism of membrane polyphosphoinositides and influx of extracellular calcium are two important activation events triggered by soluble mediators such

Table 2. Effect of lanthanum chloride on neutrophil degranulation and calcium influx

	Lactoferrin Release		Calcium Influx (CPM)	
Lanthanum Chloride (1mM)	+	-	+	-
Control	334±67	361±52	2823± 683	3251±756
Surface Bound IgG	1157±248	1321±106	3510±812	6706±1110
FMLP (10^{-6}m)	1238±316	1419±396	4219±861	7248±1217

Human neutrophls were incubated with the indicated concentration of lanthanum chloride (15min, 37°C) and then incubated in wells precoated with human IgG or BSA. In some BSA coated wells FMLP(10^{-6}M) was added. After incuabtion (15min, 37°C), lactoferrin release and calcium influx were measured as described in "Materials and Methods". Each point represents the mean and standard deviation of six determinations.

as FMLP. Solid phase IgG also appears to trigger these same two events. The drop in PIP_2 levels induced by solid phase IgG is only slightly less than the 18-30% loss reported when neutrophils are activated by FMLP (4,8,12). Since neutrophils placed in wells coated with IgG settle into the wells before engaging the non-soluble, surface bound IgG, activation may occur less uniformly than when neutrophils are exposed to a soluble ligand such as FMLP. Nonetheless, we were able to observe a 14% drop in ^{32}P incorporation into PIP_2. Similar comments can be made about the enhancement of calcium influx which is appreciable with cells incubated with IgG, but still somewhat less than with FMLP.

The mechanism by which interaction of solid phase IgG with the neutrophil results in these intracellular events is not yet known. Other soluble mediators including platelet activating factor and leukotriene B_4 activate neutrophils by a mechanism inhibitable by pertussis toxin. When these mediators are used in high concentrations, the neutrophil response is only partially inhibited by pertussis toxin, suggesting that a separate mechanism of activation may be operative (23). The lectin concanavalin A activates neutrophils in a manner which is completely insensitive to pertussis toxin (25). Surface bound IgG induced neutrophil activation is also not inhibited by pertussis toxin regardless of the amount of IgG used in the wells, suggesting that it is entirely dependent upon an alternate activation mechanism. Whether neutrophil activation by surface bound IgG is mediated through a distinct pertussis toxin insensitive G protein, as has been described in other cellular systems (25-29), is not yet clear. Gabig et al have suggested that there is a pertussis toxin insensitive G protein in the neutrophil (30).

Interestingly, soluble aggregates of IgG, in contrast to surface bound IgG, induce activation sensitive to inhibition by pertussis toxin. The mechanisms responsible for these differences are still to be delineated. Further studies on Fc receptor mediated neutrophil activation will undoubtedly provide new insights into mechanisms of neutrophil activation.

ACKNOWLEDGMENT

This research was supported by NIH grant AR20416-10.

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