EXTENDED REPORT

Insoluble and soluble immune complexes activate neutrophils by distinct activation mechanisms: changes in functional responses induced by priming with cytokines

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Background: Rheumatoid synovial fluid contains both soluble and insoluble immune complexes that can activate infiltrating immune cells such as neutrophils.

Objectives: To determine if these different complexes activate neutrophils through similar or different receptor signalling pathways. In particular, to determine the circumstances which result in the secretion of tissue damaging reactive oxygen metabolites and granule enzymes.

Methods: Blood neutrophils were incubated with synthetic soluble and insoluble immune complexes and the ability to generate reactive oxidants tested by luminescence or spectrophotometric assays that distinguished between intracellular and extracellular production. Degranulation of myeloperoxidase and lactoferrin was determined by western blotting. The roles of FcγRII (CD32) and FcγRIIIb (CD16) were determined by incubation with Fab/F(ab')₂ fragments before activation. The effect of cytokine priming was determined by incubation with GM-CSF.

Results: Insoluble immune complexes activated unprimed neutrophils, but most of the oxidants produced were intracellular. This activation required FcγRIIIb, but not FcγRII function. Soluble complexes failed to activate unprimed neutrophils but generated a rapid and extensive secretion of reactive oxygen metabolites when the cells were primed with granulocyte-macrophage colony stimulating factor (GM-CSF). This activity required both FcγRII and FcγRIIIb function. Insoluble immune complexes activated the release of granule enzymes from primed or unprimed neutrophils, but the kinetics of release did not parallel those of secretion of reactive oxygen metabolites. Only primed neutrophils released enzymes in response to soluble complexes.

Conclusions: Soluble and insoluble immune complexes activate neutrophils by separate receptor signalling pathways. Profound changes in neutrophil responsiveness to these complexes occur after cytokine priming.

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Neutrophils have a crucial role in host protection against bacterial and fungal infections and thus possess a battery of cytotoxic enzymes and associated pathways to perform this important function.¹⁻³ During phagocytosis, these cytotoxic processes are activated and delivered within intracellular phagolysosomes to effect killing, and few, if any, of these toxic molecules are released. These neutrophil derived reactive oxidants (O₂⁻, H₂O₂, ¹O₂, [•]OH, and HOCl) and granule enzymes (for example, myeloperoxidase, metalloproteinases) would cause bystander tissue damage if they were released from phagocytosing neutrophils.⁴ In contrast with this intracellular activation and containment of toxic molecules, it is also established that under appropriate conditions neutrophils can actively release large quantities of reactive oxidants and discharge the contents of their granules extracellularly. If such large scale release of these toxic molecules occurred *in vivo*, then it is likely that local antioxidants and antiproteinases would become saturated and tissue damage would ensue.

The synovial fluid of patients with active rheumatoid disease is heavily infiltrated with neutrophils that may be present at approximately 5×10⁷ cells/ml.⁵ Infiltrating neutrophils have also been detected within diseased cartilage⁶ and at the pannus/cartilage interface,⁷ which is the site of active erosions.⁸⁻⁹ Synovial fluid and cartilage from patients with rheumatoid arthritis contain large quantities of immune complexes containing IgG/immune aggregates¹⁰⁻¹¹ and these can activate the infiltrating neutrophils.¹²⁻¹³ This fluid contains two major types of immune complex, soluble and insoluble, which can be distinguished by their biochemical properties.¹⁴ Rheumatoid synovial fluid is also

a rich source of cytokines (for example, GM-CSF, interleukin 1, interferon γ) that can affect the function of infiltrating neutrophils.¹⁵⁻¹⁸ One notable effect of cytokines is their ability to "prime" neutrophil functions so that their responsiveness to stimulation is greatly enhanced.^{2, 19-20}

The aims of this study were several-fold. Firstly, we wanted to determine if soluble and insoluble immune complexes (of the type found in rheumatoid synovial fluid) stimulated neutrophils by similar or different activation pathways. Secondly, we sought to determine the effects of GM-CSF (which is found in rheumatoid synovial fluid) on neutrophil activation by these complexes. Thirdly, we determined whether these complexes could activate the secretion of reactive oxidants and granule enzymes, and whether this secretion was cytokine dependent or dependent upon the type of immune complex. Finally, we sought to identify the roles of FcγRII and FcγRIIIb, the major receptors capable of binding complexes containing IgG,²¹⁻²² in the activation processes.

MATERIALS AND METHODS

Materials

Neutrophil Isolation Medium was obtained from Cardinal Associates (Sante Fe, NM). RPMI 1640 medium was from

Abbreviations: GM-CSF, granulocyte-macrophage colony stimulating factor; HBSS, Hanks's buffered salt solution; HSA, human serum albumin; O₂⁻, superoxide anion; PI-PLC, phosphatidylinositol phospholipase C; ROI, reactive oxygen intermediate; SOD, superoxide dismutase

ICN and Fab/F(ab')₂ fragments of IV.3 (anti-CD32) and 3G8 (anti-CD16) were from Mederex (Annandale, NJ). F(ab')₂ fragments of goat antimouse IgG, luminol, isoluminol, cytochrome *c* (horse heart), human serum albumin (HSA), and the IgG fraction of rabbit antihuman HSA were from Sigma. GM-CSF was from Boehringer Mannheim (East

Sussex, UK). Phosphatidylinositol phospholipase C (PI-PLC) was from ICN Biomedicals (Irvine, CA).

Neutrophil isolation and culture

Neutrophils were purified from heparinised venous blood by a one step centrifugation step using Neutrophil Isolation Medium (Cardinal Associates), according to the manufacturer's instructions.^{23, 24} Contaminating erythrocytes were removed by hypotonic lysis with 0.1% NaCl. Purity and viability were assessed by May-Grünwald staining and trypan blue exclusion, and were >95% and >97% respectively. Purified neutrophils were suspended in RPMI 1640 medium or Hanks's buffered salt solution (HBSS) (1.2 mM CaCl₂·2H₂O, 5.4 mM KCl, 0.44 mM KH₂PO₄, 0.5 mM MgCl₂·H₂O, 0.4 mM MgSO₄·7H₂O, 0.137 M NaCl, 2.5 mM NaHCO₃, 0.33 mM Na₂HPO₄, 5.5 mM D-glucose) as indicated. They were primed by incubation with GM-CSF (50 U/ml) for 45 minutes at 37°C.

Preparation of immune complexes

Synthetic immune complexes were made using HSA and rabbit anti-HSA antibodies using the method described before.^{25, 26} The zone of equivalence (the optimal antigen:antibody ratio required for the formation of insoluble complexes) was determined by adding HSA and anti-HSA antibodies and measuring the absorption at 450 nm. Soluble immune

Figure 1 Activation of the neutrophil respiratory burst by immune complexes. (A and B) Neutrophils were incubated in HBSS at 5×10^5 cells/ml in medium that was supplemented with either 10 μ M luminol or 10 μ M isoluminol. (C) Cells were incubated at 10^6 cells/ml in HBSS supplemented with 75 μ M cytochrome *c*. Neutrophils were incubated for 45 minutes in the absence (unprimed) or presence (primed) of GM-CSF (50 U/ml). They were then stimulated with either soluble or insoluble immune complexes, as described in "Materials and methods". Typical result of at least six separate experiments are shown.

Figure 2 Effect of oxidant scavengers on chemiluminescence: activation by soluble immune complexes. GM-CSF primed neutrophils were incubated at 5×10^5 cells/ml in HBSS supplemented with either 10 μ M luminol (A) or 10 μ M isoluminol (B), in the absence or presence of SOD, catalase, or sodium azide, as described in "Materials and methods". At time zero, cells were stimulated by the addition of soluble immune complexes. Typical results of at least six separate experiments are shown.

Figure 3 Effect of oxidant scavengers on chemiluminescence: activation by insoluble immune complexes. GM-CSF primed neutrophils were incubated at 5×10^5 cells/ml in HBSS supplemented with either 10 μ M luminol (A) or 10 μ M isoluminol (B), in the absence or presence of SOD, catalase, or sodium azide, as described in "Materials and methods". At time zero, cells were stimulated by the addition of insoluble immune complexes. Typical results of at least six separate experiments are shown.

complexes were formed at fivefold the concentration of antigen required to form insoluble complexes. After formation, soluble immune complexes were briefly centrifuged (2 minutes at 13 000 g in a microfuge) to remove any contaminating insoluble complexes that might have been present. The presence of insoluble immune complexes present within preparations of soluble immune complexes and vice versa, was assessed by their characteristic activation profiles on primed or unprimed neutrophils (fig 1). Soluble complexes were formed at 120 μ g/ml antigen and 80 μ g of antibody, while insoluble complexes were formed at 20 μ g/ml antigen and 80 μ g/ml antibody. 100 μ l (8 μ g of antibody for each type of complex) added to 1 ml neutrophil suspensions at these concentrations was found to have maximal activity (data not shown). At lower concentrations of complexes, the activation kinetics were similar, but of lower magnitude.

Measurements of reactive oxidant production

For measurements of chemiluminescence, neutrophils were suspended at 5×10^5 cells/ml in HBSS medium supplemented with 10 μ M luminol or isoluminol, as indicated.^{27,28} Suspensions were stimulated by the addition of soluble or insoluble immune complexes and photon emission was followed at 37°C in an LKB 1251 luminometer. For measurements of O_2^- secretion, neutrophils were suspended at 5×10^5 cells/ml in HBSS

Figure 4 Effect of Fc γ R blocking on neutrophil activation by immune complexes. GM-CSF primed neutrophils were incubated in the absence or presence of either an F(ab')₂ fragment of 3G8 (anti-Fc γ RIIIb) or an Fab fragment of IV.3 (anti-Fc γ RII) or with both fragments, as described in "Materials and methods". Luminol chemiluminescence was then measured after the addition of either soluble (A) or insoluble (B) immune complexes. Typical results of at least six separate experiments are shown.

medium that was supplemented with 75 μ M cytochrome c .²⁹ The total volume was 200 μ l and the suspension was placed in 96 well microtitre plates. After addition of immune complexes, absorption increases at 550 nm were recorded with a Bio-Rad 3550 kinetic plate reader. Reference wells also contained 40 μ g/ml superoxide dismutase (SOD), and absorption values in these wells were subtracted to obtain SOD inhibitable O_2^- secretion. As indicated, the antioxidants SOD (to inhibit extracellular O_2^- production), catalase (to inhibit extracellular H_2O_2 production), and sodium azide (to inhibit the intracellular activity of haem proteins, particularly myeloperoxidase) were added at the indicated concentrations.³⁰⁻³²

Degranulation

After incubation of neutrophils at 1×10^6 cells/ml and stimulation with either soluble or insoluble immune complexes (as above) for the indicated times, cell suspensions were centrifuged at 3000 g for one minute. The supernatants and pellets were rapidly mixed with boiling sodium dodecyl sulphate sample buffer³³ and stored at -70°C until use. Samples were then electrophoresed on a 12% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. After Ponceau staining of the membranes to check for even protein loading of samples, membranes were probed for myeloperoxidase and lactoferrin by immunoblotting.

Figure 5 Effect of Fc γ RIIIb depletion on neutrophil activation by immune complexes. GM-CSF primed neutrophils were incubated in the absence or presence of PI-PLC as described in "Materials and methods". They were then stimulated by the addition of either soluble (A) or insoluble (B) immune complexes and luminol chemiluminescence measured. Typical results of at least six separate experiments are shown.

Immunoblots were developed using a commercial ECL kit. Densitometry of carefully exposed blots (to avoid film saturation) was performed with Image 1.44 VDM software (National Institute of Health, Bethesda, Maryland).

Treatment with PI-PLC

Primed neutrophils were incubated at 2×10^6 cells/ml in the presence of PI-PLC (0.96 U/ml) for 30 minutes at 37°C, with gentle agitation. This removed >95% of cell surface Fc γ RIIIb, as assessed by flow cytometry.^{21 22}

Receptor blocking

Primed neutrophils (5×10^6 /ml) were incubated for 15 minutes with 3 μ g/ml of the F(ab')₂ fragment of 3G8 (anti-Fc γ RIIIb) or 3 μ g/ml of an Fab fragment of IV.3 (anti-Fc γ RII), or both fragments added together to block activity of both receptors.

RESULTS

Activation of primed and unprimed neutrophils by immune complexes

The addition of insoluble immune complexes to unprimed neutrophils resulted in a slow activation of reactive oxidant

Figure 6 Activation of degranulation by immune complexes. Neutrophils were incubated in the presence and absence of GM-CSF and then stimulated for 45 minutes by the addition of either soluble or insoluble immune complexes. Cell-free supernatants were then analysed for the presence of either lactoferrin (A) or myeloperoxidase (B) by immunoblotting. In (A) samples were loaded as follows: 1, unprimed neutrophils, no additions; 2, unprimed neutrophils, soluble immune complexes; 3, unprimed neutrophils, insoluble immune complexes; 4, primed neutrophils, soluble immune complexes; 5, primed neutrophils insoluble immune complexes; 6, primed neutrophils no additions. In (B) samples were loaded as follows: 1–4, unprimed neutrophils; 5–8, primed neutrophils; 1, 5, control, no additions; 2, 6, phorbol myristate acetate (0.1 μ g/ml); 3, 7, insoluble immune complexes; 4, 8, soluble immune complexes. Typical results of at least six separate experiments are shown.

production, as detected by luminol chemiluminescence (fig 1A). When the neutrophils were primed before the addition of insoluble complexes, activation of the respiratory burst occurred more rapidly, and activity reached a higher plateau value at around seven minutes after stimulation, compared with peak activity recorded at 10–11 minutes seen in unprimed cells. In contrast, the addition of soluble immune complexes to unprimed neutrophils did not activate a respiratory burst detected by luminol chemiluminescence. However, when the cells were primed with GM-CSF before addition of soluble complexes, a rapid and transient increase in activity was seen which peaked within two minutes of addition and had declined to basal levels by seven minutes. Clearly, soluble and insoluble immune complexes activate neutrophils by different molecular processes.

Luminol chemiluminescence measures both intracellular reactive oxidants (because it can freely permeate intact neutrophils) and also oxidants that are released from the cells.^{24 27 34} It has been shown that myeloperoxidase activity is also required for the luminol reaction, together with H₂O₂ that is generated by the respiratory burst.^{35–37} Isoluminol, however, does not penetrate neutrophils and so can be used to measure only reactive oxidants that are released from activated neutrophils.^{31 32} Isoluminol was therefore used as a probe to determine extracellular release of reactive oxidants.

In contrast with the results seen with luminol, insoluble immune complexes did not activate reactive secretion in unprimed neutrophils (fig 1B). Thus the luminol chemiluminescence seen upon addition of insoluble complexes to unprimed neutrophils (fig 1A) must arise from intracellular generation of reactive oxidants. When the neutrophils were primed, the insoluble immune complexes stimulated a rapid burst of extracellular reactive oxidants, as detected by isoluminol. Thus the "extra" luminol chemiluminescence seen upon priming (fig 1A) is largely due to acquirement of the ability to secrete reactive oxidants, rather than enhanced intracellular production.

As seen with luminol chemiluminescence (fig 1A), soluble immune complexes failed to activate isoluminol chemiluminescence in unprimed cells (fig 1B). However, when the cells were primed, a rapid and transient burst of reactive oxidant secretion was detected that matched the kinetics of luminol chemiluminescence.

Figure 7 Time course of degranulation in response to immune complexes. Neutrophils were primed by incubation in the presence and absence of GM-CSF and then incubated in the absence (control, not shown) or presence of either insoluble or soluble immune complexes. At time intervals of up to 45 minutes, cell-free supernatants were tested for the presence of either myeloperoxidase (A) or lactoferrin (B) by western blotting. Typical results of at least three separate experiments are shown.

To confirm the secretion of reactive oxidants by these complexes, a completely independent assay was used. The SOD inhibitable cytochrome *c* reduction assay specifically measures O_2^- secretion from activated cells.^{24–29} Both soluble and insoluble immune complexes failed to activate the secretion of O_2^- from unprimed neutrophils (fig 1C). However, when the neutrophils were primed, then both types of immune complex activated O_2^- secretion. However, the levels of O_2^- secreted in response to soluble immune complexes were always two- to threefold higher than those released in response to insoluble complexes, confirming the data seen in fig 1B.

These data indicate that insoluble immune complexes activate intracellular reactive oxidant production in unprimed neutrophils. Presumably, this arises because the insoluble complexes are phagocytosed. When the neutrophils are primed, these insoluble complexes then activate a rapid and transient phase of reactive oxidant secretion. Soluble immune complexes, however, fail to activate unprimed neutrophils, but activate a rapid and transient burst of reactive oxidant secretion from primed cells.

Effects of scavengers

When we had established that immune complexes stimulate the production of intracellular and extracellular reactive oxidants, it was then necessary to identify the nature of these oxidants. Thus the effects of the extracellular scavengers, SOD (to scavenge extracellular O_2^-) and catalase (to scavenge extracellular H_2O_2), were determined, together with sodium azide, which inhibits the production of reactive oxidants via myeloperoxidase activity.^{28–30–32–34–38}

The production of reactive oxidants from primed neutrophils stimulated with soluble immune complexes was

virtually completely inhibited by the addition of either SOD or catalase to the extracellular medium, when either luminol (fig 2A) or isoluminol (fig 2B) was used as the chemiluminescent probe. These experiments confirm the extracellular nature of the reactive oxidants generated by stimulation of primed neutrophils with soluble immune complexes, as these scavengers cannot enter the cell. Sodium azide completely inhibited the luminol chemiluminescence, confirming the role of myeloperoxidase in the production of reactive oxidants detected by this probe.^{35–37–39} However, isoluminol dependent chemiluminescence stimulated by soluble immune complexes was largely unaffected by this myeloperoxidase inhibitor. Thus under these conditions, the reactive oxidants released by neutrophils in response to stimulation by soluble immune complexes were independent of this enzyme and likely to be largely O_2^- and H_2O_2 .

In contrast with the effects seen with soluble immune complexes, the extracellular scavengers SOD and catalase only partially decreased luminol chemiluminescence detected in primed neutrophils in response to insoluble complexes (fig 3A). This confirms that much of this chemiluminescence under these conditions is intracellular and inaccessible to these extracellular scavengers. The extracellular reactive oxidants measured by isoluminol in response to stimulation of primed neutrophils by insoluble complexes were also different from those stimulated by the soluble complexes (fig 3B). Firstly, sodium azide had a far greater inhibitory effect on the reactive oxidants secreted by insoluble complexes. This shows that myeloperoxidase derived oxidants are produced in larger amounts by primed neutrophils in response to insoluble immune complexes than in response to soluble complexes. Secondly, catalase only decreased the isoluminol chemiluminescence by about 85 (SD 3)%, ($n=6$). This indicates that H_2O_2

production in response to stimulation by insoluble complexes is lower than that stimulated by the soluble complexes.

Roles of Fcγ receptors in stimulation by soluble and insoluble complexes

The roles of FcγRII (CD32) and FcγRIIb (CD16) in activation of primed neutrophils by immune complexes was first determined in blocking studies. Primed neutrophils were pre-incubated with Fab/F(ab')₂ fragments of 3G8 (anti-FcγRIIb) and IV.3 (anti-FcγRII) before addition of complexes and measurement of luminol chemiluminescence. The production of extracellular oxidants in response to soluble immune complexes was decreased by about 53 (4)% of control values (n=6) when either FcγRII or FcγRIIb binding was blocked (fig 4A), with FcγRIIb blocking having a slightly greater inhibitory effect. However, even when binding to both receptors was blocked, there was still some detectable chemiluminescence.

In contrast, blocking FcγRII had only a slight (24 (5)%, n=6) inhibitory effect on luminol chemiluminescence stimulated in primed neutrophils by insoluble immune complexes (fig 4B). However, the response was inhibited by 78 (4)%, (n=6) when ligation to FcγRIIb was blocked. Little additional inhibitory effect was seen when binding to both receptors was prevented. The antibody used in these studies (IV.3) is reported not to bind the inhibitory receptor FcγRIIb2.⁴⁰

When surface FcγRIIb was removed from the cell surface of primed neutrophils by treatment with PI-PLC,^{21–22} activation by soluble immune complexes was completely prevented (fig 5A), whereas that stimulated by insoluble complexes was inhibited by >90% (n=6) (fig 5B). Control experiments (not shown) indicated that PI-PLC treatment did not inhibit the chemiluminescence response stimulated by phorbol myristate acetate. These experiments indicate a crucial role for FcγRIIb in binding and activation by both types of immune complex.

Degranulation

It was then necessary to determine if these complexes activated the secretion of granule enzymes and whether prior priming of the neutrophils with GM-CSF regulated this secretion. Very low levels of secretion of lactoferrin (fig 6A) or myeloperoxidase (fig 6B) were detected after 45 minutes' incubation of either unprimed or GM-CSF primed neutrophils. This low level of secretion was approximately 1–2% of the total cellular levels of these enzymes. Insoluble immune complexes could, under these experimental conditions, stimulate the release of both lactoferrin and myeloperoxidase from unprimed and primed neutrophils (fig 6). This secretion from primed or unprimed cells was time dependent, but only reached a maximum 45 minutes after addition of complexes (fig 7). Thus the kinetics of degranulation did not parallel those of secretion of reactive oxidant production (fig 1). In contrast, soluble complexes could not activate the secretion of these proteins from unprimed neutrophils, but stimulated a substantial release (approximately 50% of the total cellular levels) from GM-CSF primed cells. However, the kinetics of degranulation again did not correlate with the kinetics of secretion of reactive oxidants (fig 1).

DISCUSSION

Of all the cell types implicated in the pathology of rheumatoid arthritis, the infiltrating neutrophil has the greatest ability to inflict cellular damage.^{4 6 17} This is because the neutrophil is a highly specialised killing cell possessing a wide range of enzymes and pathways with potential for damage.⁴¹ Understanding the processes that regulate the secretion of neutrophil products at sites of inflammatory damage is important for a better understanding of disease. Identifying the molecular processes that control this secretory process may lead to the development of new therapeutic regimens for

the treatment of inflammatory diseases in which infiltrating neutrophils play an important role.

In this report, we have shown that soluble and insoluble immune complexes containing IgG activate neutrophils by completely separate processes. The major difference resides in the fact that soluble immune complexes are completely unable to stimulate the generation of reactive oxidants or degranulation in unprimed neutrophils. In contrast, insoluble complexes slowly activate a respiratory burst in unprimed neutrophils, but the oxidants that are produced are generated intracellularly. That these oxidants are intracellular in nature is confirmed by the facts that (a) they cannot be scavenged by the extracellular enzymes SOD and catalase and (b) no oxidants are detected when using the extracellular probes isoluminol or cytochrome c. It is thus likely that insoluble immune complexes are phagocytosed by unprimed neutrophils and hence the reactive oxidants are generated intracellularly, within phagolysosomes. Similarly, soluble immune complexes did not activate the secretion of myeloperoxidase or lactoferrin from unprimed neutrophils.

However, when the neutrophils were primed with GM-CSF, dramatic changes in their responsiveness to both insoluble and soluble immune complexes were detected. The most dramatic effect was the ability of soluble immune complexes (and to a lesser effect insoluble immune complexes) to activate the secretion of reactive oxidants and granule enzymes from primed neutrophils. This activation process was extremely rapid and transient, reaching a peak rate within about two minutes after stimulation, and contrasted with the slower activation of phagocytosis seen with the insoluble complexes. This priming effect was not specific to GM-CSF as a number of other proinflammatory cytokines such as interleukin 1β, tumour necrosis factor α, and interferon γ could also induce this ability in neutrophils (data not shown). As neutrophils within rheumatoid synovial fluid are primed,^{19 42–45} and as this fluid contains large amounts of these soluble complexes, this mechanism of secretion of large quantities of reactive oxidants and granule enzymes is likely to be extremely important in disease pathology.

Although insoluble immune complexes could not activate secretion of reactive oxygen intermediates (ROIs) in unprimed cells, they did activate the release of myeloperoxidase and lactoferrin from these cells. However, kinetics analysis showed that maximum enzyme release occurred 45 minutes after addition of complexes. Unprimed neutrophils did not release any enzymes (or ROIs) in response to soluble immune complexes. When the neutrophils were primed, secretion of ROIs occurred within two and eight minutes of addition of soluble and insoluble immune complexes, respectively. However, even in primed cells, maximum enzyme release was detected 45 minutes after addition and very little enzyme release was detected at earlier time points. These observations suggest that the enzyme release seen in these circumstances is not directly related to the process of secretion of ROIs. Possibly, the release of enzymes seen in these circumstances is due to discharge of the contents of vacuoles (containing granule enzymes) formed after uptake of immune complexes, rather than the result of a genuine secretory event, which generally has much faster kinetics.⁴⁶

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