Guanine Nucleotide-induced Polymerization of Actin in Electropermeabilized Human Neutrophils

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Abstract. The effects of exogenous guanine nucleotides on the polymerization of actin in human neutrophils were tested in an electropermeabilized cell preparation. Close to 40% permeabilization was achieved with a single electric discharge as measured by nucleic acid staining with ethidium bromide or propidium iodide with minimal (<2 %) release of the cytoplasmic marker lactate dehydrogenase. In addition, electropermeabilized neutrophils retained their capacity to produce superoxide anions and to sustain a polymerization of actin in response to surface-receptor dependent stimuli such as chemotactic factors. Electropermeabilization produced a rapid and transient permeabilization that allowed the entry of guanine nucleotides into the cells. GTP and, to a larger extent, its nonhydrolyzable analog guanosine 5'-O-2-thiotriphosphate (GTP[S]), induced a time- and concentration-dependent polymerization of actin, as determined by increased staining with 7-nitrobenz-2-oxa-1,3-diazoylphallacidin. The effects of the aforementioned guanine nucleotides were antagonized by GDP[S], but were insensitive to pertussis toxin. Cholera toxin potentiated to a small degree the amount of actin polymerization induced by GTP[S]. These results provided direct evidence for the involvement of GTP-binding proteins in the regulation of the organization of the cytoskeleton of neutrophils, an event that is of crucial importance to the performance of the defense-oriented functions of these cells.

The accumulation of neutrophilic polymorphonuclear leukocytes (neutrophils)1 at inflammatory sites results from the ordered and directed movement of the cells out of the circulation. The locomotory behavior of neutrophils is an essential feature of their biological functions (the body's first line of defense against foreign pathogens). Indeed, congenital defects of the contractile machinery of neutrophils leads to a severe propensity towards bacterial infections (for review see Gallin et al., 1988). The elucidation of the signaling and regulatory mechanisms controlling the movement of neutrophils, therefore, represents a central task in the comprehension of the pathophysiological basis of granulocyte functions, the first step in which is the understanding of the biochemical basis of their mechanosensory transduction apparatus (for reviews see Sha'afi and Molski, 1988; Snyderman and Verghese, 1987).

Neutrophil locomotion and phagocytosis are examples of actin-based contractile phenomena in nonmuscle cells (Stossel, 1988). Rapid and transient increases in the proportion of polymerized actin have been reported in neutrophils stimulated by chemotactic factors and other agonists (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983). This cytoskeletal reorganization is thought to play an essential role in the morphological and locomotory responses of neutrophils (Sklar et al., 1985). Despite the number of studies describing the characteristics of the chemotactic factor-stimulated increase in the amount of filamentous actin in neutrophils, it has proven difficult to identify the signals that initiate this cytoskeletal reorganization, as well as the molecular identity of the cellular constituents involved. Several lines of evidence have dissociated the polymerization of actin in neutrophils from the rise of intracellular calcium that occurs essentially concurrently (Sha'afi et al., 1986; Sklar et al., 1985; Bengtsson et al., 1986). An initiating role for the cytoplasmic acidification that accompanies neutrophil stimulation has been postulated on the basis of indirect data (Yuli and Oplatka, 1987; Molski and Sha'afi, 1987; Fancher and Naccache, 1987). However, more recent experiments have shown that the acidification response could be significantly inhibited in the absence of any effect on the stimulation of the polymerization of actin (Naccache et al., 1989).

A consistent feature of the stimulated polymerization of actin in neutrophils is its sensitivity to inhibition by pertussis toxin (Shefcyk et al., 1985; Sha'afi et al., 1986; Bengtsson et al., 1986). These results have indirectly implicated a role

1. Abbreviations used in this paper: fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; GDP[S], guanosine 5'-O-2-thiodiphosphate; GTP[S], guanosine 5'-O-2-thiotriphosphate; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase; leukotriene B4, (5S,12R)-5,12-dihydroxy-(2, E, E)-6,8,10,14-eicosatetraenoic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazolyphallacidin; neutrophil, neutrophilic polymorphonuclear leukocyte.
for guanine nucleotide-binding proteins (G proteins) in the signaling pathway(s), leading to the formation of actin filaments in neutrophils. However, pertussis toxin has been shown to affect the behavior of various cell types independently of its ADP-ribose transferase activity (Tamura et al., 1983; Rosoff et al., 1987; Banga et al., 1987; Gray et al., 1989) thereby stressing the need for confirmation of the conclusions drawn from pertussis toxin experiments by independent means. The purpose of the present study was to directly test for the involvement of G proteins in the initiation of actin polymerization. The strategy used was to examine the effects of the addition of guanine nucleotides to an electroporabilized cell preparation. The results provided direct evidence for the involvement of one or more G protein(s) in the sequence of events that leads to the initiation of actin polymerization in human neutrophils.

Materials and Methods

Peripheral blood neutrophils were purified on Ficoll-Hypaque cushions after dextran sedimentation of red blood cells. The remaining erythrocytes were lysed by hypotonic shock (30 s at 0°C temperature). The resultant suspensions of neutrophils were 97% pure and cell viability as estimated by trypan blue exclusion was >98%. Neutrophils were resuspended in calcium-free Hanks' balanced salt solution (HBSS), pH 7.4, or, when specified, in potassium buffer (110 mM KCl, 10 mM NaCl, 2 mM KH2PO4, 25 mM Hepes), pH 7.4.

Actin polymerization was measured by a modification of the 7-nitrobenz-2-oxa-1,3-diazolylphilacidin (NBD-phallacidin) staining method originally described by Howard and Oresajo (1985). When intact cells were used, 106 neutrophils were resuspended in 0.8 ml HBSS at 37°C. Electroporation was carried out in the presence or absence of the desired nucleotides by subjecting the cell suspensions to a single electric discharge of 3.75 kV/cm2 in 0.4 ms using a Gene Pulser (Bio-Rad Laboratories, Mississauga, Ontario, Canada). At the desired time after electroporation, the cells were fixed with 3.7% formalin for 15 min at room temperature and centrifuged for 15 s at 12,000 g in a microcentrifuge. The cells were then resuspended in 0.175 ml HBSS and 25 μl of a NBD-phallacidin solution in calcium-free HBSS (8.25 × 10-3 M final concentration) containing lysophosphatidylcholine (32 μg/ml final concentration) was added. This last step resulted in a fivefold increase in the concentration of NBD-phallacidin originally used by Howard and Oresajo (1985). After a 10-min incubation at room temperature in the dark, the cells were washed free of the unbound probe by centrifugation. Cell-associated fluorescence was measured (excitation and emission wavelengths were 365 and 580 nm, respectively) in a spectrofluorimeter (SLM 8000c; SLM-Aminco, Urbana, IL). The data are expressed as ratios of the fluorescence intensities of control and treated cells. Alternatively, the degree of actin polymerization was also assessed on cells stained with NBD-phallacidin as just described on a flow cytometer (Epics C; Coulter Electronics Inc., Hialeah, FL). 2,000 cells per sample were analyzed. The shift in the population distribution of the fluorescence was taken as an index of actin polymerization (Howard and Meyer, 1984).

Superoxide production was monitored as the (superoxide dismutase-sensitive) reduction of cytochrome c by a slight modification of the method described in Metcalf et al. (1986). Briefly, neutrophil suspensions (106 cells/ml) were incubated under the desired experimental conditions in the presence of 120 μM cytochrome c for 5 min at 37°C. The reactions were stopped by the addition of superoxide dismutase (final concentration, 62.5 μg/ml) and the transfer of the tubes to an ice-cold bath followed by centrifugation. The optical density of the supernatants was read at 550 nm and the amount of superoxide produced was calculated using an extinction coefficient of 21.1. The viability of permeabilized cells was assessed by the release of lactate dehydrogenase (LDH). Briefly, cells (untreated, electroporabilized, and Triton X-100-lysed; 106 cells/ml) were centrifuged down and 200 μl of the supernatants were added to 1.25 ml of phosphate buffer (0.1 M NaH2PO4, 0.1 M Na2HPO4, pH 7.35, enriched with 0.14 mg/ml NADH). The reactions were started by the addition of 50 μl of sodium pyruvate solution and the reduction of the NADH was monitored for 1 min by a UVVIS spectrophotometer at 340 nm. The data are expressed as a percent of the total LDH content as determined after Triton X-100 (0.1%) lysis of the cells samples.

The permeabilization of the cells was evaluated in two ways. First, untreated, electroporabilized, and Triton X-100-lysed cells were stained with 75 μM ethidium bromide for 20 min at room temperature. Fluorescence intensities (excitation and emission wavelengths were 365 and 580 nm, respectively) of control and permeabilized cells were compared with that of Triton X-100 (0.1%)-lysed cells in the fluorimeter. Internal controls were run to correct for the direct effects of Triton X-100 on the fluorescence of ethidium bromide. Second, untreated, permeabilized, and Triton X-100-lysed cells were stained with 80 μg/ml propidium iodide for 5 min at room temperature before being analyzed by flow cytometry using a flow cytometer (Epics C; Coulter Electronics Inc.). 5,000 cells per sample were analyzed. The extent of permeabilization was quantified by comparison of the fluorescence of electroporabilized with Triton X-100-lysed cells.

Dextran T-500 and Ficol-Hypaque were purchased from Pharmacia Fine Chemicals (Dorval, Quebec, Canada). Lysophosphatidylcholine, sodium pyruvate solution, NADH*, Triton X-100, ethidium bromide, propidium iodide, and cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-phallacidin was from Molecular Probes Inc. (Junction City, OR). Formalin was obtained from Fisher Scientific Co. (Quebec, Quebec, Canada). Pertussis toxin was purchased from List Biological Co. (Campbell, CA).

Results

Characterization of the Electroporabilization

The exposure of neutrophils to a single electric discharge (1,500 V, 25 μF) induced a rapid permeabilization as evidenced by the staining of nucleic acids by ethidium bromide (Table I). Since intact cells are impermeable to this dye (Gomperts, 1983), the increased fluorescence of the cell suspension after the electric discharge was an index of permeabilization. The procedure used allowed the penetration of enough dye to stain close to 40% of the nucleic acid content of the cells. Cell permeabilization was also verified by flow cytometry using propidium iodide to examine for potential nonhomogeneities in the response of the cells to the electric discharge. Control cells displayed only minimal levels of fluorescence as expected from intact cells excluding the dye. Electroporation resulted in the detection of a single peak of fluorescence that included >95% of the cells (Fig. 1), indicating that practically the entire cell population responded to the permeabilization procedure.

The effects of the electroporation on the viability of the cells was assessed by measuring the leakage of LDH. As shown in Table I, a single electric discharge increased the amount of LDH recovered in the supernatants of the cells by only ~2% in excess of that recovered from control, untreated cells. Subjecting neutrophils to two or three electric discharges increased the degree of staining with ethidium bromide; however, increasing levels of cell damage, as indicated by LDH leakage as well as progressive loss of cell responsiveness (ability to produce superoxide anions in response to formylmethionyl-leucyl-phenylalanine [fMet-Leu-Phel]), were also caused by multiple electric discharges (Table I). The degrees of permeabilization and release of lactate dehydrogenase induced by three electric discharges corresponded closely to those reported by Grinstein and Furuya (1988) using a similar procedure.

The ability of neutrophil suspensions to respond to chemotactic factors was also examined as a test of the functional viability of the electroporabilized cells. As shown in Table I, stimulation of control and electroporabilized cells (one discharge) with fMet-Leu-Phel or PMA resulted in the formation of equivalent amounts of superoxide. Furthermore, fMet-Leu-Phel induced similar increases in the relative amounts
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The effects of guanosine 5'-O-2-thiotriphosphate (GTP[S]) on actin polymerization in control and electropermeabilized cells were investigated; the results of these studies are summarized in Table II. The responses to the chemotactic factor fMet-Leu-Phe are shown for comparative purposes. The addition of GTP[S] to nonpermeabilized cells was without effect on the level of polymerized actin in neutrophils. The level of fluorescence detected in the electropermeabilized cells diminished slightly (∼20%) subsequent to the electric discharge, probably from the combination of a small loss of cells, some leakage of globular actin, and potential effects of the treatment on the actin polymerization/depolymerization equilibrium (results not shown). GTP[S] increased the amount of F-actin in electropermeabilized cells by >60%, an effect of a similar magnitude to that elicited by optimal concentrations of chemotactic factors. Furthermore, fMet-Leu-Phe increased the fluorescence ratio to about the same extent in control, untreated cells as it did in cells that had been subjected to one electric discharge.

The effects of GTP[S] on the fluorescence of NBD-phallacidin-stained neutrophils were also analyzed by flow cytometry (Fig. 2). Neither the addition of GTP[S] to nonpermeabilized cells nor the electroporation of cells in the absence of nucleotides altered the position of the peak of fluorescence. The latter was, however, shifted to the right (indicating increased fluorescence) in cells electropermeabilized in the presence of 1 mM GTP[S], a result consistent with the presence of larger amounts of polymerized actin in these cells. There were no indications of inhomogeneity in the response of the cell population to the guanine nucleotide.

The dependence of the actin polymerization response in untreated and electropermeabilized cells on the ionic composition of the suspending buffer was studied. Equivalent increases in the NBD-phalloidin fluorescence ratios caused...
Table II. Effect of Guanine Nucleotides on the Level of Actin Polymerization in Human Neutrophils

| Stimuli* | Electroporation† | Actin polymerization‡ (fluorescence ratios) |
|----------|------------------|--------------------------------------------|
| None     | -                | 1.0                                        |
| fMET-LEU-PHE | -               | 1.44 ± 0.05 (25)                           |
| GTP [S]  | -                | 1.03 ± 0.05 (3)                            |
| None     | +                | 1.0                                        |
| fMET-LEU-PHE | +               | 1.29 ± 0.05 (4)                            |
| GTP [S]  | +                | 1.64 ± 0.07 (31)                           |

* The concentrations of fMet-Leu-Phe and GTP [S] were 10⁻⁷ and 10⁻³ M, respectively. The stimulation times were 30 s and 3 min, respectively.
† Electroporation was performed where indicated by plus signs as described in Materials and Methods (one discharge).
‡ Actin polymerization was measured as described in Materials and Methods. Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.

The effects of ATP, GTP, GDP, and guanosine 5′-O-2-thiodiphosphate (GDP[S]) on the level of polymerization of actin in electropermeabilized human neutrophils were compared with those of GTP[S] (Fig. 6). Of these nucleotides, only GTP and GTP[S] elicited significant increases in fluorescence. The response to GTP was, however, significantly smaller than that induced by an equimolar amount of its non-hydrolyzable analog GTP[S]. Furthermore, the simultaneous inclusion of equimolar amounts of GTP[S] and GDP[S] during the electroporation decreased the response to GTP[S] by ~50%. The mean actin polymerization indices were 1.64 ± 0.07 (n = 31) for GTP[S] and 1.33 ± 0.03 (n = 3) for GTP[S] plus GDP[S] (p < 0.01). It should be noted that the experimental protocol precluded preincubation with GDP[S].

Sensitivity of the Actin Polymerization Response of Electropermeabilized Neutrophils to Pertussis and Cholera Toxin

The effect of pertussis and cholera toxin on the ability of either fMet-Leu-Phe and (5S,12R)-5,12-dihydroxy-(2,E,E,2)-6,8,10,14-eicosatetraenoic acid (leukotriene B₄) or GTP[S] to induce actin polymerization in intact and permeabilized neutrophils, respectively, was examined next (Fig. 7). In these

Concentration Dependence of the Polymerization of Actin Induced by GTP[S]

The stimulation of the polymerization of actin induced by GTP[S] in electropermeabilized human neutrophils was concentration dependent (Fig. 5). A threshold concentration of 0.1 mM GTP[S] was necessary for the detection of a significant response to the nucleotide. The magnitude of the response increased up to the highest concentration tested (1 mM).
Figure 3. Kinetics of the increase in actin polymerization elicited by GTP[S] in electropermeabilized human neutrophils. GTP[S] (1 mM) was added to the cells immediately before electroporation. The experimental conditions are detailed in Materials and Methods. The open squares represent the results of the addition of GTP[S] to electropermeabilized cells. The filled square represents the effect of the addition of GTP[S] to nonpermeabilized cells. Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate (the error bar of the 30-s time points lies within the size of the symbol).

experiments, the freshly isolated cells were incubated for 3 h at 37°C with 0.5 μg/ml of either pertussis or cholera toxin before stimulation. Pertussis toxin virtually abolished the responses of intact cells to the two chemotactic factors, while cholera toxin was without any inhibitory activity. Furthermore, pertussis toxin also inhibited the polymerization of actin induced by the chemotactic factors in electropermeabil-

Discussion

The study of the potential role of G proteins in the mediation of the effects of neutrophil agonists has relied to a great extent on the evidence derived from the use of bacterial toxins. Although it is known they specifically ADP-ribosylate different Nucleotides

Figure 6. Nucleotide specificity of the stimulation of actin polymerization in electropermeabilized human neutrophils. The cells were electropermeabilized in the presence of 1 mM of the indicated nucleotides. Asterisks and double asterisks indicate significant differences from control (p < 0.05 and p < 0.01, respectively). Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in triplicate.
The electroporation procedure described above yielded a viable and transiently permeabilized preparation of neutrophils into which otherwise impermeable molecules such as guanine nucleotides could penetrate. The low level of LDH leakage and the ability of the cells to sustain an oxidative burst and a polymerization of actin in response to chemotactic factors after the electroporation procedure indicated that few cytoplasmic constituents, be they small or macromolecules, were lost from the cells during the permeabilization phase and that the transduction apparatus of the cells was intact. These characteristics distinguish the present experimental conditions from those used by Grinstein and Furuya (1988) which rendered neutrophils unresponsive to chemotactic factors (superoxide production) unless exogenous NADPH was added, thereby indicating that a significant (and uncontrolled) leakage of intracellular components had taken place. The gentleness of this procedure, as well as its ease of reproducibility, are major advantages over the more commonly used detergent permeabilization protocols in studies that do not require permanent access to the cytoplasmic milieu. Indeed, the permeabilization induced by a single electric discharge was transient, lasting <3 min.

The addition of nonhydrolyzable guanine nucleotides (such as GTP[S]) to electropememabilized neutrophils resulted in a time- and concentration-dependent increase in the amount of polymerized actin. The magnitude of this response is similar to that induced by optimal concentrations of the chemotactic factors fMet-Leu-Phe and leukotriene B4 (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983; Shefcyk et al., 1985). The time course of the effect of GTP[S] on actin polymerization is slower and more sustained than that observed in response to chemotactic factors (White et al., 1983; Rao and Varani, 1982; Howard and Meyer, 1984; Fechheimer and Zigmond, 1983; Shefcyk et al., 1985). These kinetic differences reflect the higher efficiency of the receptor-induced GDP/GTP exchange as compared with that elicited by the direct addition of the nucleotide (Gilman, 1984). GTP[S] was without effect when added to nonpermeabilized cells thereby emphasizing the requirement for access to the cytoplasmic compartment for the nucleotide to exert its effects. Though the concentrations of GTP[S] required are relatively high when compared with those necessary with isolated membrane preparations, the use of similar ranges of concentrations of guanine nucleotides (up to 1 mM) has been reported in various permeabilized cell preparations including neutrophils (Barrowman et al., 1986; Huang and Devanney, 1986; Scott and Dolphin, 1987; Knight and Baker, 1985). This need probably arises from the relatively low efficiency of incorporation of the guanine nucleotides dictated in part by the gentleness of the permeabilization procedure used in this study. The nucleotide specificity of the actin response (only GTP[S] and GTP are

### Table III. Effect of Pertussis Toxin on the Polymerization of Actin Induced by Chemotactic Factors in Intact and Electropermeabilized Neutrophils

| Stimuli       | Treatment* | Control (stimulation ratios) | Pertussis toxin   |
|---------------|------------|------------------------------|-------------------|
| fMet-Leu-Phe  | −          | 1.50 ± 0.13 (3)              | 1.07 ± 0.07 (3)   | <0.05 |
|               | +          | 1.45 ± 0.20 (3)              | 1.12 ± 0.05 (3)   | <0.08 |
| Leukotriene B4| −          | 1.44 ± 0.17 (3)              | 1.04 ± 0.14 (3)   | <0.07 |
|               | +          | 1.51 ± 0.06 (3)              | 0.98 ± 0.02 (3)   | <0.01 |

* Treatment refers to the exposure to the electric discharge.

† Mean ± SEM of the number of experiments, indicated in parenthesis, each carried out in duplicate.
active in this assay) is consistent with the hypothesis that a G protein is involved. This is further supported by the inhibition of the effects of GTP[S] induced by GDP[S], a common feature of G protein-activated systems. Furthermore, the larger effects induced by GTP[S] as compared with GTP imply that the signals initiated upon the interaction of the nucleotide with its target protein are terminated upon the hydrolysis of the terminal phosphate by the GTPase inherent to the α subunit of G proteins. These various features of the actin polymerization response to GTP[S] are characteristic of G protein-mediated events. It remains to be determined whether the GTP[S]-induced polymerization of actin in permeabilized cells is related to the solubilization of actin and myosin from rabbit neutrophil membranes described by Huang and Devanney (1986).

The differential sensitivities of the effects of GTP[S] and of the chemotactic factors to the bacterial toxins raised difficult questions that are, however, not unique to this experimental system. The effects of none of the three stimuli were inhibited by cholera toxin; in fact, a potentiation of the actin polymerization response to the guanine nucleotide, the basis of which is presently unknown, was observed. On the other hand, the effects of fMet-Leu-Phe and of leukotriene B4 in intact as well as electroporpermeabilized neutrophils, but not those of GTP[S], were quite sensitive to inhibition by pertussis toxin. The chemotactic factor-induced increase in the incorporation of actin in the Triton X-100-insoluble pellet of rabbit neutrophils has previously been shown to exhibit a similar sensitivity to the two toxins (Shefky et al., 1985). In contrast, several reports indicate that the various effects of GTP[S] are not affected by pertussis toxin. In neutrophils, Barrowman et al. (1986) observed that the potentiation of the degranulation induced by GTP[S] was not inhibited by pertussis toxin. In membranes prepared from human neutrophils (Smith et al., 1987) or differentiated HL-60 cells (Okajima et al., 1985), pertussis toxin inhibited fMet-Leu-Phe-induced augmentation of phospholipase C activity, but not the stimulation of IP3/IP formation elicited by GTP or GTP[S]. Furthermore, the time course of exocytosis in response to GTP[S] remained unaffected in pertussis toxin-treated mast cells, whereas the stimulation by compound 48/80 of the secretion was completely abolished (Lindau and Nube, 1987). These results can be explained in one of two ways.

First, several pools of G proteins may exist. These may represent distinct entities, one or more of which is insensitive to pertussis toxin although capable of transducing the signal induced by GTP[S] into an increase in the amount of polymerized actin. Alternatively, a proportion of a single G protein population may be, because of its membrane location and/or accessibility, resistant to ribosylation by the bacterial toxin. Second, GTP[S] may be able to bypass the functional inhibition induced by ribosylation of the G proteins. The examination of this hypothesis depends on the precise identification of the relevant G protein and of the effector system and the subsequent direct examination of the functional competence of the ribosylated G protein.

These various hypotheses cannot be differentiated between at present. It should be noted, however, that the insensitivity of the effects of GTP[S] on the polymerization of actin to pertussis toxin are consistent with previous suggestions that there may be a toxin-resistant G protein in neutrophils (Barrowman et al., 1986). Whether this postulated pertussis toxin-insensitive G protein is coupled to the chemotactic factor receptors or plays other, as yet undefined, roles in neutrophil activation remains to be investigated.

In summary, the results presented in this communication indicated that GTP-binding proteins are involved in the regulatory mechanisms involved in the reorganization of the neutrophils' cytoskeleton. This conclusion is based on the finding that the direct introduction of guanine nucleotides induces, in a specific manner, a polymerization of actin in human neutrophils. Furthermore, the electropermeabilization procedure described herein should find a variety of applications since it provides a convenient means of introducing impermeable molecules into functionally responsive cells.

The authors wish to thank Ms. Caroline Gilbert for her expert help. This is part of the work submitted for the fulfillment of an M. S. thesis (S. Therrien) in the Department of Medicine at Université Laval, Ste Foy, Québec, Canada.

Supported in part by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Arthritis Society of Canada and by fellowships from the Fonds de la Recherche en Santé du Québec (P. H. Naccache) and the Arthritis Society (S. Therrien).

Received for publication 28 February 1989 and in revised form 19 May 1989.

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