Evidence for specific annexin I-binding proteins on human monocytes

Nicolas J. GOULDING*§, Luying PAN†‡, Kathleen WARDWELL†, Veronica C. GUYRE† and Paul M. GUYRE†

*Department of Biochemical Pharmacology, The Medical College of St. Bartholomew's Hospital, London, U.K. and †Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756, U.S.A.

Recombinant human annexin I and a monoclonal antibody specific for this protein (mAb 1B) were used to investigate surface binding of this member of the annexin family of proteins to peripheral blood monocytes. Flow cytometric analysis demonstrated trypsin-sensitive, saturable binding of annexin I to human peripheral blood monocytes but not to admixed lymphocytes. A monoclonal antibody that blocks the anti-phospholipase activity of annexin I also blocked its binding to monocytes. These findings suggest the presence of specific binding sites on mono-

cytes. Furthermore, surface iodination, immunoprecipitation and SDS/PAGE analysis were used to identify two annexin I-binding proteins on the surface of monocytes with molecular masses of 15 kDa and 18 kDa respectively. The identification and characterization of these annexin I-binding molecules should help us to better understand the specific interactions of annexin I with monocytes that lead to down-regulation of pro-inflammatory cell functions.

INTRODUCTION

Annexin I is a member of a family of widely distributed calcium-binding proteins which have been studied in diverse biological systems, mainly with regard to their interactions with phospholipids and membranes, their potential role in exocytosis and their regulation of cell growth and differentiation [1–5]. At least 13 distinct proteins are known to constitute the annexin family of proteins. All have a common core structure consisting of four or eight repeats of a highly conserved 70–80-amino-acid unit which contain the calcium- and phospholipid-binding activities, but the N-terminal region of each member is unique and it is this heterogeneity which probably confers the various biological properties that have been reported for the different annexins [6–8].

Annexin I was initially considered to be a secreted, glucocorticoid-induced protein which specifically inhibited phospholipase A₂ (PLA₂) (reviewed in [9]). Subsequent studies raised questions over the glucocorticoid induction of annexin I production [10,11], its secretion from cells and the physiological importance of PLA₂ inhibition [12–15]. This last point has raised most controversy, with the majority of evidence supporting a substrate depletion model of PLA₂ inhibition rather than direct enzyme interaction. More recently, however, glucocorticoid regulation of annexin I production both *in vivo* and *in vitro* [16–19], and selective secretion of annexin I by prostate epithelial cells [20] have been firmly established. Moreover, purified annexin I and derived peptides have been shown to exert acute anti-inflammatory effects [21,22] and to inhibit brain ischaemic damage [23] in *in vivo* models. Annexin I also appears to mimic many effects of glucocorticoids on leucocytes including inhibition of superoxide generation [24], chemotaxis/production of eicosanoids [25] and binding of IgG to Fc receptors [26]. In each of these cases, however, the mechanisms of action remain unclear and there are little data available comparing the effects of recombinant and native proteins.

These latter observations in particular suggested to us that leucocytes may contain surface binding sites which may mediate the effects of exogenous annexin I. Since it proved technically impossible to radiolabel annexin I and maintain biological activity, we used a monoclonal antibody specific for annexin I to evaluate its binding to cells. In this report, we extend our preliminary finding that annexin I binds specifically to monocytes, but not to lymphocytes, in healthy human subjects [27].

EXPERIMENTAL

Blood cell separation and purification

Mononuclear cells were freshly isolated from citrated (0.38%) peripheral blood of normal human donors. Comparison of anticoagulants showed similar results for heparin- and EDTA-anticoagulated blood, with annexin I binding consistently about 20% higher when citrate was used (results not shown). Both Ficoll-Hypaque M-85 single-step gradient [28] and buffy coat [29] cell preparations were used with identical results. After washing isolated cells with RPMI-1640 (Gibco), cells were resuspended in Hepes-buffered RPMI-1640 containing 0.2 g/l CaCl₂ and 2 mg/ml BSA (1640 + + +). In experiments to determine the sensitivity of annexin I binding to protease pretreatment, cells at a density of 1 × 10⁶/ml were incubated with a final concentration of 250 µg/ml trypsin (Sigma) for 40 min at 37 °C with gentle rotation. Proteolysis was then quenched by a 3-fold dilution of the reaction mixture in RPMI 1640 containing 10% (v/v) fetal bovine serum. The cells were subsequently washed three times in protein-free RPMI-1640 before being resuspended in 1640 + + + and tested for annexin I binding.

Immunofluorescence staining and flow cytometry

Since it has proved technically impossible to radiolabel annexin I for binding studies and maintain biological activity, we used an indirect immunofluorescence assay in which the binding of

Abbreviations used: PLA₂, phospholipase A₂; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

‡ Present address: Department of Pediatric Oncology, Dana Farba Cancer Research Institute, Boston, MA 02115, U.S.A.

§ To whom correspondence should be addressed.

recombinant human annexin I (Biogen, Cambridge, MA, U.S.A.) was detected with a specific monoclonal antibody (mAb), mAb 1B [30]; Biogen), which shows no cross-reactivity with other annexins. Recombinant human annexin I was produced in *Escherichia coli* and purified to > 99% as previously described [31]. Preparations consisted of 85% intact N-terminus, with 15% of material lacking N-terminal amino acids 1–10. Endotoxin levels were below 50 pg/ml. Recombinant human protein was used in these studies rather than native mammalian protein due to its ready availability and comprehensive characterization. Cells (5×10^5) were incubated in 1640 + + + medium with recombinant annexin I in 96-well flat-bottomed tissue-culture plates (Costar) as a mixture (lymphocytes and monocytes) in a total volume of 60 μ l for 1 h at 4 °C unless otherwise indicated. Cells were then washed twice with 200 μ l of Dulbecco's phosphate-buffered saline containing 2 mg/ml BSA and 0.2 g/l CaCl_2 (PBS-BSA-Ca) to remove free annexin I, and then incubated with 20 μ g/ml mAb 1B for 1 h at 4 °C. They were then washed three times with PBS-BSA-Ca and incubated with fluorescein isothiocyanate (FITC)-labelled goat (Fab')₂ anti-(mouse IgG) (Caltag, S. San Francisco, CA, U.S.A.) for 1 h at 4 °C. Monocytes and lymphocytes were examined by flow cytometry, gated by forward and 90° light scatter, and by lineage-specific surface markers. Analysis was performed on a Becton Dickinson Facsan flow cytometer. Cell-associated mean fluorescence intensity was converted into second antibody molecules bound per cell according to a calibration curve using standard fluorescence beads from Flow Cytometry Standards Co. (San Juan, PR, U.S.A.) [32].

Radioimmunoprecipitation

To test the hypothesis that annexin I is binding to a specific protein or proteins on the cell surface, monocytes from normal donors were subjected to surface iodination and immunoprecipitation with recombinant annexin I and mAb 1B. Monocytes were freshly isolated from monocyte-enriched leukophoresis packs using Ficoll-Hypaque gradient separation and cold aggregation [33]. After overnight culture in RPMI-1640 containing 10% fetal bovine serum, cells were labelled with ¹²⁵I using the lactoperoxidase method [34], and lysed with PBS containing 0.5% Nonidet P-40, 0.2 g/l CaCl_2 , 0.1 g/l MgCl_2 , and a cocktail of proteinase inhibitors as previously described [33]. After preclearing with Protein A-Sepharose three times, or anti-PLA₂ following Protein A-Sepharose three times, lysates were mixed with recombinant human annexin I for 1 h, then mAb 1B was added. Samples were rotated overnight at 4 °C, then mixed with Protein A-Sepharose for 4 h. After washing the Protein A-Sepharose with lysis buffer six times, samples were eluted into 2 × sample buffer plus 8% (v/v) 2-mercaptoethanol by boiling for 3 min or into 50 μ l of 10 mM EGTA in PBS, then added to an equal volume of 2 × sample buffer plus 8% 2-mercaptoethanol. Samples were analysed by 10% reducing SDS/PAGE [35].

RESULTS

Characteristics of annexin I binding to monocytes and lymphocytes

Binding sites for annexin I were found on human peripheral blood monocytes but not lymphocytes, when both cell types were incubated together with recombinant human annexin I at either 4 °C or 37 °C (Figure 1). Binding of annexin I to monocytes from different healthy individuals varied over a wide range as has previously been described [36]. No significant binding to lympho-

Figure 1 Time course of recombinant human annexin I binding to human monocytes

Buffy coat leucocytes were isolated from citrated blood as described in the Experimental section, and then incubated at 4 °C (●) or 37 °C (▲) for the times indicated. Incubation mixture was 60 μ l of 1640 + + + containing 5×10^5 mixed leucocytes and 3 μ g/ml recombinant human annexin I. Binding was detected by indirect immunofluorescence plus quantitative flow cytometry as described elsewhere [28] and is expressed as secondary antibody molecules bound per cell. Data are from one individual.

Figure 2 Dose-dependent binding of annexin I to human monocytes

Buffy coat leucocytes were isolated from citrated blood as described in the Experimental section, and then incubated at 4 °C (●) or 37 °C (▲) with increasing concentrations of recombinant human annexin I. Binding was detected as described in Figure 1. Data are from one individual.

cytes was observed. The experiment in Figure 1 was repeated using leucocytes from three different donors, and on each occasion the binding at 37 °C declined at ≥ 2 h. Therefore, subsequent experiments entailed 1 h incubations unless noted otherwise. The binding of annexin I was saturable as shown in Figure 2. In five experiments, half-saturable binding ranged from 0.5 to 3 μ g/ml (13.3–80 nM) annexin I. When cells were allowed to bind annexin I at 4 °C for 1 h and were then incubated further, loss of bound annexin I occurred over a 2 h period at 37 °C, but not at 4 °C (Figure 3). In order to examine the specificity of this assay for surface-associated annexin I, we compared the binding of the annexin I-specific antibody (mAb 1B) with that of three other monocyte-specific mAbs, using cells either pretreated or not pretreated with annexin I. Figure 4 shows that binding of mAb 1B was negligible in the absence of annexin pretreatment of cells, and that mAb binding to other surface antigens was unaffected by annexin pretreatment.

Figure 3 Annexin I binding to monocytes is lost during incubation at 37 °C

Peripheral blood leucocytes were isolated as previously described and incubated with 20 μ g/ml recombinant human annexin I at 4 °C in RPMI + + + medium for 60 min as described in the Experimental section. Cells were then washed three times in RPMI + + + and incubated for a further 180 min at either 4 °C (●) or 37 °C (▲). Binding was detected at selected time points using the method already described.

Figure 4 mAb 1B specifically recognizes bound annexin I on human cells

Isolated blood leucocytes were incubated in the presence (▨) or absence (■) of recombinant human annexin I for 60 min at 4 °C. Unbound annexin I was removed by washing and cells were incubated with 20 μ g/ml mAb specific for either annexin I (1B); type-I Fc gamma receptor, CD64 (32.2); class I major histocompatibility complex (MHC) (w6.32) and class II MHC (IV.A12). After reaction for 60 min and washing, antibody binding sites were detected using FITC-conjugated goat (Fab')₂ anti-(mouse IgG) and flow cytometry (see the Experimental section).

Annexin I binding is calcium-dependent and trypsin sensitive

Because annexin I requires Ca²⁺ for biological activity, we examined the importance of Ca²⁺ for its binding to leucocytes. Figure 5 shows that if cells were washed with PBS lacking Ca²⁺ after incubation with annexin I, or if they were incubated with annexin I in the presence of 10 mM EDTA, the binding of annexin I was abolished. In addition, mAb 1A, which neutralizes the anti-PLA₂ activity of annexin I [30], also markedly reduced the binding of annexin I to monocytes even when Ca²⁺ was present (Figure 5). We next determined whether a trypsin-sensitive protein was involved in the binding of annexin I to leucocytes. Table 1 shows that preincubation of monocytes with 250 μ g/ml trypsin resulted in an 81 % decrease in annexin I/mAb 1-B binding. Expression of the CD15 carbohydrate surface antigen recognized by mAb PM81 was resistant to trypsinization as expected, whereas binding of mAb Mac 2.48 to a 155 kDa trypsin-sensitive surface antigen [33] showed a 70 % reduction with trypsin treatment.

Figure 5 Calcium-dependence and specificity of annexin I binding to human leucocytes

Peripheral blood leucocytes were isolated as previously described and incubated with 20 μ g/ml recombinant human annexin I at 4 °C in RPMI medium for 60 min as described in the Experimental section. Cells were reacted with annexin I alone (dark hatched bar) or annexin I in the presence of a specific mAb 1A which neutralizes annexin I activities (light hatched bar). Annexin I-bound cells were also washed in calcium-free PBS (closed bar) or in the presence of 10 mM EDTA (open bar).

Table 1 Trypsin sensitivity of annexin I/mAb 1B binding to human peripheral blood monocytes

Monocytes were protease-pretreated according to the protocol described in the Experimental section. After washing, cells were incubated with either 20 μ g/ml annexin I, followed by 20 μ g/ml mAb 1B or 20 μ g/ml mAb PM81 recognizing the trypsin-resistant CD15 surface antigen or 20 μ g/ml mAb Mac 2.48, a glucocorticoid-induced monocyte marker known to be trypsin-sensitive. Antibody binding was detected using FITC-conjugated goat (Fab')₂ anti-(mouse IgG) and flow cytometry (see the Experimental section). Results are expressed as antibody binding sites per cell $\pm$ S.D. of triplicates.

mAb	Pretreatment		Percentage of control
	Control	Trypsin (250 μ g)	
Annexin I/1B	44 700 $\pm$ 4900	8000 $\pm$ 700	19
PM81 (CD15)	27 800 $\pm$ 3600	41 400 $\pm$ 1100	149
Mac 2.48	5000 $\pm$ 700	1400 $\pm$ 300	30

Effect of annexin V on annexin I binding

We sought to determine the specificity of binding to monocytes within the annexin family of proteins by studying the binding of annexin V to freshly isolated peripheral blood leucocytes. Increasing concentrations (0–2 μ g/ml) of recombinant human annexin V (Biogen, Cambridge, MA, U.S.A.) were incubated with leucocytes as described in the Experimental section. Binding was detected using an mAb specific for annexin V (Biogen, Cambridge, MA, U.S.A.) and secondary FITC-labelled goat (Fab')₂ anti-(mouse IgG) (Caltag, S. San Francisco, CA, U.S.A.). In three separate experiments, no significant binding of annexin V was observed to monocytes or lymphocytes (results not shown). However, a 10-fold excess (15 μ g/ml) of either recombinant human, human placental or bovine placental annexin V were equally effective at blocking annexin I (1.5 μ g/ml) binding to monocytes by > 90 % in three donors (Figure 6).

Annexin I-specific binding proteins on monocytes

The finding that recombinant human annexin I binds to human monocytes in a saturable and trypsin-sensitive manner suggested that annexin I could be binding to a specific protein site on

Figure 6 Annexin V inhibits annexin I binding to monocytes

Peripheral blood leucocytes were isolated as previously described and incubated with either medium alone (closed bar), 15 µg/ml recombinant human annexin V (open bar), human placental annexin V (stippled bar) or bovine placental annexin V (hatched bar) at 4 °C in RPMI medium for 10 min as described in the Experimental section. Annexin I (1.5 µg/ml) was subsequently added and left to react for a further 60 min at 4 °C before annexin I binding was measured as described previously.

Figure 7 Annexin-binding proteins characterized by immunoprecipitation and SDS/PAGE

Autoradiograph of reducing SDS/PAGE showing immunoprecipitation with: lane 1, annexin I and an irrelevant mAb RPC5; lane 2, annexin I and mAb 1B; lane 3, mAb 1B alone; lane 4, mAb w6/32 anti-class I MHC antigen; lane 5, solid-phase bead fraction after annexin I and mAb 1B binding and elution with EGTA; lane 6, annexin I and mAb 1B after 3-fold preclearing with anti-PLA₂ antibody; lane 7, anti-PLA₂ antibody; lane 8, supernatant fraction after annexin I and mAb 1B binding and elution with EGTA. Two other independent experiments yielded similar results.

human monocytes and not merely to membrane anionic phospholipids. Analysis of surface radioiodinated human monocytes by SDS/PAGE and autoradiography demonstrated that recombinant annexin I, in conjunction with mAb 1B, immunoprecipitated two molecules with molecular masses of 15 kDa and 18 kDa respectively (Figure 7). mAb 1B did not precipitate any radiolabelled molecules in the absence of exogenous annexin I, indicating that the 15 kDa and 18 kDa molecules bound specifically to the added annexin I and were not proteolytic fragments of endogenous annexin I, which might also be recognized by mAb 1B. After washing immunoprecipitate-bound Protein A-Sepharose with PBS containing EGTA, these two

molecules were found in the supernatant, whereas the recombinant annexin I and mAb 1B were still in the Sepharose fraction.

DISCUSSION

The lack of radiolabelled annexin I that retains biological activity has, until now, hindered quantitative studies of the binding of annexin I to the surface of intact cells. We have used a different approach, employing the binding of recombinant annexin I to cells followed by an annexin I-specific mouse mAb that had been previously determined not to alter the biological activity of the molecule [30]. Fluoresceinated goat F(ab')₂ anti-(mouse IgG) plus quantitative flow cytometry was then used to estimate the relative number of annexin I molecules bound to monocytes and lymphocytes which had been exposed to annexin I together in the same tube. Significant, saturable binding of annexin I to monocytes, but not to admixed lymphocytes, suggested that the annexin I was not merely coating membrane phospholipid in these cell types but was binding in a more specific manner. This conclusion was further supported by the trypsin sensitivity of binding, by inhibition of binding by mAb 1A which blocks the anti-phospholipase activity of annexin I, and by the isolation by immunoprecipitation of two specific binding proteins. Interestingly, the ability of leucocytes to bind annexin I is lost when cells are isolated from sites of inflammation [37]. This could be due to the release of proteases at these sites which are cleaving the binding proteins.

Specific and functionally relevant annexin I binding of this nature is supported by prior studies. Blackwell et al. [38] observed that in the guinea pig perfused-lung system, PLA₂ activity was inhibited almost as soon as lipocortin infusions were begun and declined shortly after infusions were terminated. This could be due to rapid binding of lipocortin to the cell surface, resulting in a signal to inhibit PLA₂ activity. Hirata et al. [39] also reported that glucocorticoid-induced inhibition of neutrophil chemotaxis was completely reversed if the neutrophils were digested with pronase first, suggesting that pronase-sensitive membrane protein(s) were important in mediating lipomodulin effects. Annexin I binding to human neutrophils has proved to be enigmatic. Initial studies appeared to demonstrate saturable binding similar to monocytes [27], but subsequent studies using highly purified preparations of neutrophils have failed to show significant binding (results not shown).

We recently reported that recombinant human annexin I inhibits IgG binding to leucocyte Fc receptors. This effect only occurred in cells with annexin I-binding capacity. Annexin I inhibited IgG binding to monocytes, which have annexin I high binding, but it did not inhibit IgG binding to lymphocytes or the cell lines K562, U937 or HL60, which do not bind significant amounts of annexin I [26]. Moreover, although N-terminal-deleted annexin I bound to cells, it did not interfere with IgG binding to Fc receptors. Taken together, these results indicate that specific binding of annexin I to human leucocytes by regions independent of the N-terminus, plus some interaction(s) requiring the N-terminus, are necessary for inhibition of leucocyte function [40,41].

The initial binding of annexin I may involve the well-known association of annexins with anionic phospholipids [6,13]. This interaction has been studied extensively for annexin V. Rao et al. [42] have demonstrated saturable, phospholipid-dependent binding of annexin V to a human ovarian carcinoma cell line. Binding of annexin V to phosphatidylserine exposed on the outer membrane leaflet of cells is calcium dependent [43] and has been used as a sensitive assay for detecting the onset of programmed

cell death [44,45]. We were unable to demonstrate annexin V binding to freshly isolated monocytes, a result which would have been predicted by the lack of exposed phosphatidylserine residues on the external surface of the plasma membrane of these cells [44]. Therefore the observation that excess annexin V blocked annexin I binding to monocytes was unexpected. At this stage, we can only speculate that soluble annexin I and V heteromultimers may possibly be being formed, with the annexin I component of the complex losing its affinity for the binding site. There is good evidence for the occurrence of multimeric forms of annexins [46,47] but the physiological relevance of these complexes remains to be determined. There is also some evidence for interaction of annexin V with cell-surface proteins in certain cell types. Annexin V has been reported to bind specifically to a 50 kDa protein expressed on the surface of human placental fibroblasts [48]. Further studies are now required to determine the identity and physiological importance of the cell-surface proteins that bind annexin I and annexin V relative to their interaction with phospholipids.

The tissue distribution of annexin I may provide a clue to the circumstances under which binding to leucocytes plays an important physiological role. The concentration of annexin I is less than 50 ng/ml in the blood [49], but the protein concentration may be as high as 100 µg/ml in epithelial [50] and endothelial [51] environments, as it is in prostatic secretions [20]. Thus, the dose dependency for binding (Figure 2) predicts that leucocytes in blood will be devoid of surface annexin I, but will have the potential to bind annexin I upon extravasation into tissues. The marked glucocorticoid effect on tissue levels of annexin I [18] suggest that the amount of this annexin that will be available to bind to tissue-infiltrating leucocytes is regulated, at least in part, by prior exposure to glucocorticoid hormones [52].

In summary, we have identified saturable, trypsin-sensitive and calcium-dependent binding sites for annexin I that are selectively expressed on human monocytes but not lymphocytes. We have also identified two monocyte surface proteins to which annexin I binds specifically. The identification of these annexin I-binding proteins on human leucocytes provides a new direction for investigation into the mechanism of the extracellular effects of annexin I. Further characterization of the nature of the specific plasma membrane-associated annexin I-binding proteins on human leucocytes, together with studies of signal transduction and cell function after annexin binding, could provide a basis for greater understanding of the mechanisms of action and physiological importance of the lipocortin/annexin family of proteins.

We thank Dr. Jeffery Browning of Biogen Research Corp., Cambridge, MA, U.S.A. for supplying recombinant human annexin I and mAbs 1A and 1B used in this study. This work was supported in part by grant G0082 from the Arthritis and Rheumatism Council for Research, U.K. and by National Institutes of Health grant DK 33100 and the Norris Cotton Cancer Center Support Core Grant CA 23108.

REFERENCES

- Blackwood, R. A. and Ernst, J. D. (1990) *Biochem. J.* **266**, 195–200
- Schlaepfer, D. D. and Haigler, H. T. (1990) *J. Cell Biol.* **111**, 229–238
- Creutz, C. E. (1992) *Science* **258**, 924–931
- Raynal, P. and Pollard, H. B. (1994) *Biochim. Biophys. Acta* **1197**, 63–93
- Ernst, J. D., Mall, A. and Chew, G. (1994) *Biochem. Biophys. Res. Commun.* **200**, 867–876
- Schlaepfer, D. D. and Haigler, H. T. (1987) *J. Biol. Chem.* **262**, 6931–6937
- Klee, C. B. (1988) *Biochemistry* **27**, 6645–6653
- Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K. S., Pratt, D. et al. (1988) *J. Biol. Chem.* **263**, 10799–10811
- Flower, R. J. (1988) *Br. J. Pharmacol.* **94**, 987–1015
- Bienkowski, M. J., Petro, M. A. and Robinson, L. J. (1989) *J. Biol. Chem.* **264**, 6536–6544
- Isacke, C. M., Lindberg, R. A. and Hunter, T. (1989) *Mol. Cell. Biol.* **9**, 232–240
- Northup, J. K., Valentine-Braun, K. A., Johnson, L. K., Severson, D. L. and Hollenberg, M. D. (1988) *J. Clin. Invest.* **82**, 1347–1352
- Davidson, F. F., Dennis, E. A., Powell, M. and Glenney, Jr., J. R. (1987) *J. Biol. Chem.* **262**, 1698–1705
- Hayashi, H., Owada, M. K., Sonobe, S., Domae, K., Yamanouchi, T., Kakunaga, T., Kitajima, Y. and Yaoita, H. (1990) *Biochem. J.* **269**, 709–715
- Kim, K. M., Kim, D., Park, Y. M., Kim, C.-K. and Na, D. S. (1994) *FEBS Lett.* **343**, 251–255
- Solito, E., Raugel, G., Melli, M. and Parente, L. (1991) *FEBS Lett.* **291**, 238–244
- Solito, E., Nuti, S. and Parente, L. (1994) *Br. J. Pharmacol.* **112**, 347–348
- Vishwanath, B. S., Frey, F. J., Bradbury, M., Dallman, M. F. and Frey, B. M. (1992) *Endocrinology* **130**, 585–591
- Goulding, N. J., Godolphin, J. L., Sharland, P. R., Peers, S. H., Sampson, M., Maddison, P. J. and Flower, R. J. (1990) *Lancet* **335**, 1416–1418
- Christmas, P., Callaway, J., Fallon, J., Jones, J. and Haigler, H. T. (1991) *J. Biol. Chem.* **266**, 2499–2507
- Cirino, G., Flower, R. J., Browning, J. L., Sinclair, L. K. and Pepinsky, R. B. (1987) *Nature (London)* **328**, 270–272
- Cirino, G., Peers, S. H., Flower, R. J., Browning, J. L. and Pepinsky, R. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3428–3432
- Strijbos, P. J. L. M., Hardwick, A. J., Relton, J. K., Carey, F. and Rothwell, N. J. (1992) *Am. J. Physiol.* **263**, E632–E636
- Maridonneau-Parini, I., Errasta, M. and Russo-Marie, F. (1989) *J. Clin. Invest.* **83**, 1936–1940
- Fradin, A., Rothhut, B., Poincelot-Canton, B., Errasta, M. and Russo-Marie, F. (1988) *Biochim. Biophys. Acta* **963**, 248–257
- Goulding, N. J. and Guyre, P. M. (1993) *Biochem. Biophys. Res. Commun.* **192**, 351–358
- Goulding, N. J., Luying, P. and Guyre, P. M. (1990) *Biochem. Soc. Trans.* **18**, 1237–1238
- Ferrante, A. and Thong, Y. H. (1978) *J. Immunol. Methods* **24**, 389–393
- Hoffman, R. A., Kung, P. C., Hansen, W. P. and Goldstein, G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4914–4917
- Pepinsky, R. B., Sinclair, L. K., Douglas, I., Liang, C.-M., Lawton, P. and Browning, J. L. (1990) *FEBS Lett.* **261**, 247–252
- Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P. and Browning, J. L. (1986) *Nature (London)* **320**, 77–81
- LeBouteiller, P. P., Mishal, Z., Lemonnier, R. A. and Kourilsky, F. M. (1983) *J. Immunol. Methods* **61**, 301–315
- Morganelli, P. M. and Guyre, P. M. (1988) *J. Immunol.* **140**, 2296–2304
- Manjunath, R., Graziano, R. F. and Green, W. R. (1986) *J. Immunol.* **136**, 2271
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Goulding, N. J., Luying, P., Jefferiss, C. M., Rigby, W. F. C. and Guyre, P. M. (1992) *Arthritis Rheum.* **35**, 1395–1397
- Perretti, M., Flower, R. J. and Goulding, N. J. (1993) *Biochem. Biophys. Res. Commun.* **192**, 345–350
- Blackwell, G. J., Carnuccio, R., Di Rosa, M., Flower, R. J., Parente, L. and Persico, P. (1980) *Nature (London)* **287**, 147–149
- Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1980) *Biochemistry* **19**, 2533–2536
- Hoekstra, D., Buist-Arkema, R., Klappe, K. and Reutelingsperger, C. P. (1993) *Biochemistry* **32**, 14194–14202
- Wang, W. and Creutz, C. E. (1994) *Biochemistry* **33**, 275–282
- Rao, L. V. M., Tait, J. F. and Hoang, A. D. (1992) *Thromb. Res.* **67**, 517–531
- Trotter, P., Orchard, M. A. and Walker, J. H. (1995) *Biochem. J.* **308**, 591–598
- Koopman, G., Reutelingsperger, C. P. M., Kuijten, G. A. M., Keehnen, R. M. J., Pals, S. T. and van Oers, M. H. J. (1994) *Blood* **84**, 1415–1420
- Homburg, C. H. E., De Haas, M., von dem Borne, A. E. G. Kr., Verhoeven, A. J., Reutelingsperger, C. P. M. and Roos, D. (1995) *Blood* **85**, 532–540
- Pepinsky, R. B., Sinclair, L. K., Chow, E. P. and O'Brine-Greco, B. (1989) *Biochem. J.* **263**, 97–103
- Lueke, H., Chang, B. T., Mailliard, W. S., Schlaepfer, D. D. and Haigler, H. T. (1995) *Nature (London)* **378**, 512–515
- Sen-Majumdar, A., Murthy, U. and Das, M. (1986) *Biochemistry* **25**, 627–633
- Uemura, K., Inagaki, H., Wada, Y., Nakanishi, K., Asai, K., Kato, T., Ando, Y. and Kannagi, R. (1992) *Biochim. Biophys. Acta* **1119**, 250–255
- Fava, R. A., McKanna, J. and Cohen, S. (1989) *J. Cell. Physiol.* **141**, 284–293
- Romisch, J., Schuler, E., Bastian, B., Burger, T., Dunkel, F.-G., Schwin, A., Hartmann, A. A. and Paques, E.-P. (1992) *Blood Coag. Fibrinolysis* **3**, 11–17
- Goulding, N. J. and Guyre, P. M. (1992) *Immunol. Today* **13**, 295–297