Biochimica et Biophysica Acta, 673 (1981) 286—302
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BBA 29524

LIPOSOME UPTAKE BY HUMAN LEUKOCYTES

ENHANCEMENT OF ENTRY MEDIATED BY HUMAN SERUM AND AGGREGATED IMMUNOGLOBULINS

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(Received May 13th, 1980)
(Revised manuscript received September 26th, 1980)

Key words: Liposome uptake; Immunoglobulin G; Enzyme replacement therapy; (Human leukocyte)

Summary

The entry of immunoglobulin-coated liposomes into human leukocytes bearing Fc receptors was evaluated using two methods: (i) the cellular association of liposomal markers (^{3}H-labelled phosphatidylcholine, lipid phase; [^{14}C]inulin, aqueous phase), and (ii) the ultrastructural cytochemistry of cells following incubation of cells with liposomes containing a cytochemical marker (horseradish peroxidase) in the aqueous spaces. The entry of liposomes into a cell population composed predominantly of neutrophils was linear for 10—15 min and was mediated by an active process that appeared to be both energy- and surface-dependent. This uptake could be largely inhibited by incubation at 0°C, and by exposure to glutaraldehyde, iodoacetamide, *N*-ethylmaleimide, and an excess of aggregated immunoglobulins. Entry into cells of multilamellar liposomes was saturable, displaying affinity constants of 1.1 and 1.7 mM. Ultrastructural analysis of the heterogeneous leukocyte population showed that monocytes took up liposomes more actively than neutrophils and lymphocytes. Moreover, liposomes were almost always found within the leukocytes, rather than adherent to the outer plasma membrane. The relative avidity of mono-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IgG, immunoglobulin G; Fc, complement binding portion of the immunoglobulin molecule; PMN, polymorphonuclear.

cytes was confirmed by comparing the uptake of radiolabelled liposomes by a 'pure' neutrophil population, a 'mixed' neutrophil population, and a 'mononuclear cell' population. Precoating liposomes with high molecular weight aggregates of human immunoglobulin G resulted in enhanced serum-independent uptake. The fraction of aggregated immunoglobulin G which was most effective in provoking uptake of coated liposomes also stimulated the greatest amount of lysosomal enzyme secretion. These data suggest that the interaction (precoating) of liposomes with either high molecular weight aggregates of immunoglobulin G or with human serum enhances their subsequent uptake by human leukocytes.

Introduction

Liposomes can act as vectors not only for a variety of biologically active substances captured in their internal, aqueous compartments, but also for similar materials which have become associated with their lipid membranes [1—7]. Multilamellar liposomes may interact with cells by four mechanisms: (i) they may undergo endocytosis and enter the lysosomal apparatus [2,6,7]; (ii) they may undergo fusion with the plasma membrane of target cells, leading to the internalization of a liposome minus its outermost bilayer [2,8]; the intracytoplasmic liposome may either be degraded in situ or may undergo a secondary fusion event with membrane-bounded organelles; (iii) they may bind to cell surfaces as a result of electrostatic attraction [2,9], and may subsequently be internalized by either fusion or endocytosis; and (iv) liposomal membrane constituents may undergo lipid exxhange with cell membranes [10]. These pathways are not mutually exclusive. Their relative contributions to the total interaction are determined by the liposome composition, the type of recipient cell, and the incubation conditions.

If liposome-encapsulated substances are to be delivered successfully to selected organ(s) or tissue(s), targeting techniques must be devised in order to bypass the accumulation of liposomes at undesirable sites and to optimize the delivery to specific cells. In one such technique, liposomes can be coated with aggregated immunoglobulins (a surrogate for immunoglobulin configuration in immune complexes) to serve as a cell surface ligand [6,7]. The results of studies [11—14] of the interaction of immunoglobulins with liposomes may be summarized as follows: (i) heat-aggregated, rather than native immunoglobulins, preferentially coat liposomes [11]; (ii) heat-aggregated immunoglobulin G caused an apparent perturbation of a hydrophobic electron spin resonance probe within multilamellar vesicle bilayers, whereas native immunoglobin G was ineffective; the aggregated immunoglobulin-G-induced spin label mobility was reflected functionally by an increase in the passive diffusion rate of chromate anion previously sequestered within the anionic vesicles [12]; (iii) the conditions of immunoglobulin aggregation affect both the size of the aggregates and their frequency of occurrence: heat-aggregated (62°C, 30 min) human immunoglobulin G may be chromatographically resolved into Fractions I, II, and III, having sedimentation coefficients of 24—280 S, 14—23 S, and 3—13 S, respectively [13,14]; (iv) the ability of immunoglobulin G aggregates to bind a

spin-labelled fatty acid (16-doxyl stearic acid) is a function of the degree of aggregation (hydrophobicity), binding most tightly to the larger aggregate species; the apparent dissociation constants (K_d) for Fractions I, II, and III are $8.99 \cdot 10^{-6}$ M, $37.5 \cdot 10^{-6}$ M, and $204 \cdot 10^{-6}$ M, respectively [13,14]. These findings suggest that heat-aggregation induces a conformational change in the Fc portion of the immunoglobulin G molecules, permitting them to interact with liposomal bilayers by promoting hydrophobic associations with the acyl chains of phospholipids. In addition, aggregated immunoglobulins should form lattices in which the key Fc regions are disposed both toward the interior of the outermost lamellae of multilamellar liposomes and toward the surrounding medium. These act as ligands for the Fc receptors of leukocytes, thereby provoking endocytosis of the aggregated immunoglobulin-coated liposomes [6,7]. This communication is an extension of these studies.

Materials and Methods

Preparation of liposomes. Multilamellar liposomes (phosphatidylcholine/dicetyl phosphate/cholesterol, 70 : 20 : 10) were prepared with ^{3}H-labelled phosphatidylcholine as the lipid phase marker and [^{14}C]inulin (M_r = 5000) as the aqueous phase marker. The sources of the lipids and the method of liposome preparation were detailed previously [4]. Hand-shaken liposomes, formed in a swelling solution containing 6.67 mg inulin/ml, entrap 8.2 ± 2.3 μl per μmol lipid ($\bar{X}$ ± S.D., n = 19) within their aqueous spaces; this trapped volume also corresponds to 55 ± 15 ng inulin/nmol lipid [4].

Isolation of polymorphonuclear leukocyte and mononuclear cell populations. Both mononuclear and polymorphonuclear cells were isolated from heparinized (10 U/ml) blood of normal human donors. Polymorphonuclear leukocytes were separated by either of two methods: a dextran-sedimentation procedure [15] or the Hypaque/Ficoll gradient procedure of Boyum [16]. Contaminating erythrocytes were lysed in 0.15 M ammonium chloride. In order to work with a cell population devoid of polymorphonuclear leukocytes, we used the mononuclear cell fraction from Hypaque/Ficoll gradients. Cells were suspended in Dulbecco's phosphate-buffered saline modified to contain 0.6 mM $CaCl_2$/1.0 mM $MgCl_2$/10 mM Hepes/5 mM D-glucose (pH 7.4). Cellular viability was 95—99% as determined by exclusion of trypan blue. Differential counts were made on Wright-Giemsa stained smears.

Liposome uptake into leukocytes. Cells, suspended at a final concentration of $(5—15) \cdot 10^6$/ml, were incubated together with liposomes (1—2 μmol phospholipid/ml) usually in the presence of human serum (10%, v/v), at 37°C in a shaking water bath. Equal volumes of the cell suspension (maintained on ice for up to 2 h) and of the liposome suspension (preincubated at 37°C) were mixed and incubated together. At various time intervals, quadruplicate 200-μl aliquots of the incubation mixture were withdrawn. The cells were separated from the liposomes by rapid centrifugation (at $8000 \times g$ for 2 min in an Eppendorf Model 3200 microfuge) through a silicone layer (G.E. Versilube F-50; specific gravity, 1.05) and into 0.6 M sucrose (specific gravity, 1.10); the cells passed through the silicone layer whereas the liposomes remained in the supernatant [17]. Liquid scintillation counting procedures for the analysis of radioactive

inulin and phospholipid markers in the cell pellets were described previously [18]; the counts were corrected for background and channel spillover. Trapped water in the cell pellet was determined after mixing aliquots of the cell suspension with non-encapsulated [^{14}C]inulin. The zero-time values for uptake of liposomal markers were determined by mixing ice-cold aliquots of cells and radioactively labelled liposomes and centrifuging within 10 s. These zero-time values, which reflect both trapped water and rapid binding of liposomes to cell surfaces, were subtracted from subsequent uptake values.

Aggregation of immunoglobulins and interaction with liposomes. Both human and rabbit immunoglobulin G (Miles Labs) were dissolved in phosphate-buffered saline (17 mg/ml) and heated at 62°C for 15 min; the resulting aggregates were fractionated on a Bio-Gel A-5M (Bio-Rad) (1.6 × 40 cm) column, patterned after methods reported previously [13,14].

The liposome peak fractions (five 2-ml aliquots) eluted from Sepharose 2B (1.6 × 40 cm) columns were pooled (5.3 ± 0.3 μmol liposomal lipid/ml). The pooled liposomes were agitated by sonication for 15 s in a 50 W bath-type sonicator (Heat Systems Ultrasonics, Plainview, NY) and immediately mixed with the various immunoglobulin G fractions. The resultant suspensions of coated liposomes were incubated for 20 min at 37°C and for 2 h at 23—26°C prior to use.

Association of immunoglobulin G with liposomes. Two techniques were employed. In the first method, 2-ml samples of liposomes that had been incubated together with aggregated rabbit immunoglobulin G (at least 17 μg protein/ml) were rechromatographed on a Sepharose 2B (1.6 × 40 cm) column. The effluents were assayed for lipid content either by relating absorbancy at 410 nm to that of standard dilutions of the original preparations or by quantification of the ^{3}H-labelled phosphatidylcholine content by comparison with the radioactivity in the initial preparation; protein content was determined by the method of Lowry et al. [19]. In the second method, immunoglobulin-G-coated liposomes were immunoprecipitated with a specific antiserum. Liposomes were coated with aggregated rabbit immunoglobulin G (32 μg protein/μmol lipid). Each of three 240 μl aliquots (0.15 μmol lipid/ml) were diluted up to a volume of 5000 μl with phosphate-buffered saline and then received either 1, 5 or 10 units of goat antibody to rabbit γ-globulin (1 unit will precipitate the γ-globulin in 200 μl of 2% normal rabbit serum during 6 h). These mixtures were then centrifuged at 20°C with a total pelleting force equalling 430 000 × g · min. The liposomal suspensions and supernatants were analyzed before and after the centrifugations for the presence of the liposomal markers ^{3}H-labelled phosphatidylcholine and [^{14}C]inulin.

Lysosomal and non-lysosomal enzyme release from leukocytes. Aliquots (1 ml) of cell suspensions containing from (2—4) · 10^{6} cells were dispensed into tubes, and preincubated with cytochalasin B (5.0 μg/ml) for 10 min to optimize enzyme release by 'reverse endocytosis' [15]. Cells were incubated at 37°C for 15 min in either the absence (control) or the presence of various phagocytic stimuli. These stimuli included either aggregated immunoglobulins (preparation described above) or serum-treated zymosan. Zymosan (Sigma) (10 mg/ml) was opsonized for 30 min at 37°C and then washed with phosphate-buffered saline [15]. Incubations were terminated by centrifugation for

10 min at 1500 × *g*, at 2°C. The cellular supernatants were assayed for the lysosomal enzyme β-glucuronidase as well as for the cytoplasmic marker enzyme lactate dehydrogenase [15]. The total enzyme content of these cells was measured in replicate aliquots after the cells were lysed by the addition of Triton X-100 detergent (0.2%, v/v).

Electron microscopy. Cells were fixed and processed for electron microscopy by the method of Graham and Karnovsky [20] to localize horseradish peroxidase reaction product. Cell pellets were embedded in Spurr's epoxy (Polysciences, Inc.), sectioned with a diamond knife on a Porter-Blum MB2 ultramicrotome, and viewed in a Zeiss EM 9S electron microscope.

Results

Time course of liposomal uptake by leukocytes. The time courses for the cellular association of the individual radiolabels of doubly radioactively labelled liposomes (Fig. 1) are parallel; however, the linear uptake by cells of [^{14}C]-inulin in the aqueous phase and of ^{3}H-labelled phosphatidylcholine in the lipid phase is rapid for the first 10—15 min and then decreases with time. The initial rate of inulin uptake by the polymorphonuclear leukocyte-enriched population over the first 10 min was enhanced by 7.5-fold over the control value when the inulin was encapsulated within liposomes; at 60 min, encapsulated inulin uptake was 5.5-fold greater than for free inulin. Unentrapped inulin presumably enters the cells via pinocytosis.

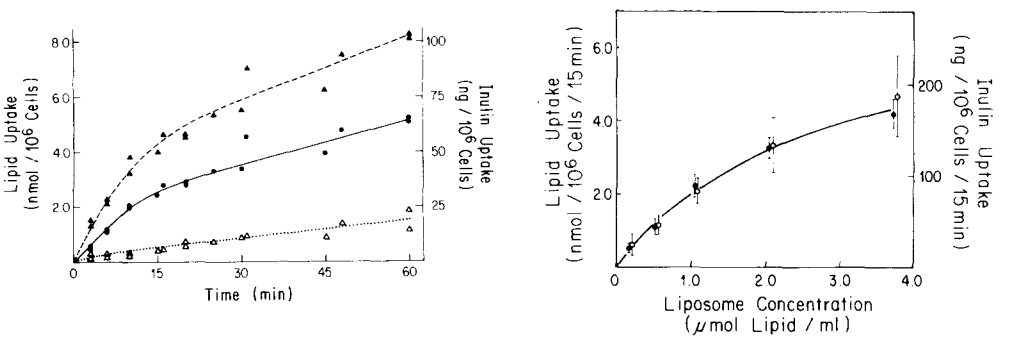


Fig. 1. Time course of liposomal uptake by human polymorphonuclear leukocytes. Polymorphonuclear leukocytes ((17—21) · 10^6/ml), isolated by the dextran sedimentation method, were incubated in the presence of serum (10%, v/v) with uncoated doubly radioactively labelled liposomes (2.6 μmol lipid/ml). ▲, uptake of liposomal aqueous space marker [^{14}C]inulin (ng/10^6 cells); ●, uptake of liposomal lipid phase marker ^{3}H-labelled phosphatidylcholine (nmol/10^6 cells). Replicate aliquots of cells were incubated with a quantity of non-entrapped, soluble radioactively labelled inulin (△) equalling that present within liposomes. The curves are a composite of two separate experiments; each point represents the mean of two determinations.

Fig. 2. Concentration dependence of liposomal uptake by human polymorphonuclear leukocytes. Polymorphonuclear leukocytes ((12—21) · 10^6/ml), isolated by the dextran sedimentation method, were incubated for 15 min in the presence of serum (10%, v/v) with five concentrations of uncoated, doubly radioactively labelled liposomes ranging from 0.15 to 3.75 μmol lipid/ml. ○, rate of uptake of liposomal aqueous phase marker [^{14}C]inulin (ng/10^6 cells/15 min); ●, rate of uptake of liposomal lipid phase marker ^{3}H-labelled phosphatidylcholine (nmol/10^6 cells per 15 min). Each plotted value represents the mean ±S.E. of four experiments, each of which was performed in quadruplicate.

Kinetics of liposomal entry into cells. The initial rates of multilamellar liposome uptake were determined at multiple liposomal concentrations ranging from 0.15 to 3.75 μmol lipid/ml incubation mixture. When the cellular uptake of both liposomal [^{14}C]inulin and ^{3}H-labelled phosphatidylcholine is plotted as a function of liposomal concentration (Fig. 2), the resulting curves are hyperbolic, indicating that saturation is being approached. This was confirmed by a double reciprocal (Lineweaver-Burk) transformation of the data which yielded linear curves for both inulin and lipid uptake rates ($r^2 \geqslant 0.97$), having negative X-axis intercepts; liposomal affinity constants (K_m) of 1.1 and 1.7 mM were determined for the liposomal aqueous space marker (inulin) and for the liposomal lipid phase marker (phosphatidylcholine), respectively. Therefore, the association of multilamellar liposomes with leukocytes is mediated by a saturable process.

Effects of metabolic inhibitors on liposome-cell interaction. The following series of experiments were performed in order to ascertain whether the entry process for liposomes is an active process. The results, summarized in Table I, are expressed in terms of the percentage of the control uptake of phospholipids and inulin after 15 min of incubation.

Incubation of cells with liposomes at 0°C, which should prevent the internalization of vesicles but not their surface adsorption, or in the presence of the protein cross-linking agent glutaraldehyde resulted in a greater than 88% reduction in the uptake of aqueous and lipid phase liposomal markers. The association of liposomes with cells, therefore, occurs largely through an active process. Cytochalasin B, an agent which inhibits microfilament function, and colchicine, a compound which interferes with microtubule function, had only a small

TABLE I

EFFECT OF INHIBITORS ON UPTAKE OF MULTILAMELLAR LIPOSOMES BY HUMAN LEUKOCYTES

Leukocytes, isolated by the dextran sedimentation method, were preincubated in the presence of inhibitors for 15 min at 37°C, and incubated with doubly radioactively labelled liposomes in the continued presence of inhibitors and serum (10%, v/v) for 15 min. Each experimental value (n) is the mean of four replicate determinations.

Treatment	Liposome uptake (% control ± S.E. (n))	
	Phospholipid	Inulin
Control	100	100
2-Iodoacetamide ($5 \cdot 10^{-3}$ M)	48 ± 7 (6) *	49 ± 10 (6) *
Sodium fluoride ($5 \cdot 10^{-2}$ M)	85 ± 20 (6)	85 ± 18 (6)
Sodium azide ($5 \cdot 10^{-3}$ M)	97 ± 8 (6)	88 ± 7 (6)
N-Ethylmaleimide ($1 \cdot 10^{-3}$ M)	24 ± 4 (6) *	19 ± 2 (6) *
Colchicine ($1 \cdot 10^{-5}$ M)	100 ± 7 (6)	80 ± 5 (6) *
Cytochalasin B (5 μg/ml)	86 ± 8 (6)	75 ± 8 (6) *
Glutaraldehyde (0.5% (v/v))	12 ± 1 (5) *	2 ± 1 (4) *
0°C	5 ± 2 (3) *	−4 ± 1 (4) *
Heat-aggregated IgG (1 mg/ml)	35 (1)	5 (1)
Zymosan, serum-treated (1 mg/ml)	114 (1)	62 (1)
Latex particles (0.15% (v/v))	84 (1)	51 (1)

* vs. control; $P < 0.05$, Student's t-test.

effect on liposome association with the cells. However, at the concentration used (5 μg/ml), cytochalasin B was previously shown to inhibit only 20—30% of the phagocytic entry of Oil Red-O droplets by human polymorphonuclear leukocytes [21]. Sodium fluoride, which is an inhibitor of anaerobic glycolysis, and sodium azide, which inhibits oxidative phosphorylation, were also ineffective in blocking liposome uptake. However, iodoacetamide, which blocks the anaerobic glycolytic pathway at a different site than sodium fluoride, reduced liposome uptake by 50%, and *N*-ethylmaleimide reduced uptake by 80%, supporting the possibility that uptake is mediated to some extent by an energy-dependent mechanism. In addition, both iodoacetamide and *N*-ethylmaleimide are sulfhydryl reagents and may non-specifically alkylate accessible sulfhydryl groups, thereby interfering with vesicle internalization by altering the surface properties of plasma membranes. The lack of literature describing the effects of these inhibitory agents upon both cellular metabolic functions and cellular membranes prevents us from defining specific mechanisms of liposome-cell interaction. Our data does, however, support, although not conclusively, the notion that uptake is mediated by an active, energy-dependent process.

Studies were conducted to further determine the role of the membrane surface properties in the uptake of liposomes. The three established phagocytic stimuli, immunoglobulin G aggregates, serum-treated zymosan and polystyrene latex particles, interact with human leukocytes via Fc receptors, C3b receptors, and 'nonspecific' receptors, respectively [22]. Exposure of the cells to an excess of unfractionated immunoglobulin G aggregates before and during incubation with liposomes reduced the uptake of liposomal lipid and inulin by 65 and 95%, respectively; serum-treated zymosan and latex particles had a smaller inhibitory effect (Table I). These results are consistent with the hypothesis that the uptake of liposomes by human phagocytes, in the presence of serum (10%, v/v), is mediated to some extent via surface Fc receptors. When the Fc receptors of the cells are saturated by an excess of immunoglobulin G aggregates free in the medium (1 mg/ml), they fail to recognize, bind to, and internalize liposomes.

Interaction of ligand (immunoglobulin G) with liposomes. We have attempted to enhance the amount of immunoglobulin G associated with liposomes in order to stimulate phagocytosis and provoke vesicle uptake by cells. Liposomes were treated with heat-aggregated rabbit immunoglobulin G (unfractionated), and rechromatographed on Sepharose 2B columns. Exposing the lipid vesicles (6.4 μmol lipid/ml) to an immunoglobulin G concentration of 100 μg/ml resulted in the association (co-elution) of approximately 17 μg protein/μmol lipid, and comprising 85% of the total eluted protein. Increasing the immunoglobulin G concentration over a 4-fold range did not result in any further association of the protein with the vesicles. Since this binding was determined by gel chromatography under non-equilibrium conditions, we cannot exclude the possibility that more immunoglobulin could associate with the vesicles under appropriate conditions. However, we may assume that the protein found to bind to the liposomes represents either a lower limit to the possible binding, or an association of a high-affinity component of the unfractionated immunoglobulin G population.

To elucidate further the interaction and association of immunoglobulins with the liposomes, we measured the disruption of liposomes coated with unfractionated immunoglobulin G aggregates (rabbit) following the addition of an antiserum to rabbit immunoglobulin G. As summarized in Table II, liposomal disruption was assayed on the basis of the dissociation of the aqueous marker, inulin, from the radioactively labelled phospholipid phase following centrifugation. Uncoated liposomes alone, or following exposure to an anti-γ-globulin antiserum, could be sedimented by centrifugation, leaving only 22—32% of their radioactively labelled phospholipid and inulin in the supernatant; coated liposomes alone behaved identically. In contrast to these internal controls, however, when immunoglobulin-coated liposomes were exposed to the anti-γ-globulin antiserum, they released 90% of their entrapped inulin into the supernatant as did vesicles that were disrupted by sonication (4 min). These results suggest that the interaction of antibody molecules (antiserum) with liposome-bound antigens (immunoglobulin G aggregates) disrupted the liposomes.

We have also studied the ability of immunoglobulin G aggregates of differing size and with different binding affinities to stimulate phagocytosis and provoke the cellular uptake of liposomes. Consequently, both human and rabbit immunoglobulin G preparations were heat-aggregated and then fractionated on Bio-Gel A-5M columns. The elution profiles for the human and rabbit preparations (Fig. 3), although treated identically, are distinctly different. This variance may be attributed to a species difference in the response to heat aggregation. The human immunoglobulin preparation eluted with the appearance of a high molecular weight peak (fraction I) and a low molecular weight peak (fraction III); the leading shoulder of the low molecular weight peak was designated fraction II. In contrast, rabbit immunoglobulin G exhibited propor-

TABLE II

SEDIMENTATION OF LIPOSOMAL RADIOLABEL MARKERS AS A FUNCTION OF IMMUNOGLOBULIN COATING AND SUBSEQUENT IMMUNOPRECIPITATION

Total pelleting force equals 430 000 × g · min. Values represent mean ± S.D. (n = 3). Multilamellar liposomes, doubly labelled with ^{3}H-labelled phosphatidylcholine (lipid phase) and [^{14}C]inulin (aqueous phase), were pretreated (coated) with heat-aggregated immunoglobulin G (rabbit), treated with various concentrations of an antiserum to rabbit γ-globulin, and were sedimented by centrifugation. Goat antiserum to rabbit γ-globulin; 1 unit (U) will precipitate the γ-globulin in 200 μl of 2% normal rabbit serum during 6 h.

Treatment			Percentage of total liposomal markers remaining in supernatant	
Pretreatment	Post-treatment		Phospholipid	Inulin
Heat-aggregated IgG-coating	Anti-IgG antiserum	Sonication		
0	0	0	32 ± 2	32 ± 2
0	10 U	0	30 ± 2	30 ± 2
+	0	0	22 ± 3	22 ± 4
+	1 U	0	38 ± 2	56 ± 3
+	5 U	0	44 ± 4	56 ± 5
+	10 U	0	46 ± 3	90 ± 5
+	0	4 min	68 ± 4	90 ± 4

tionately less of a fraction I peak. These results are in agreement with earlier experiments [13,14] demonstrating that human immunoglobulin G fractions I, II and III have molecular sizes of 24—280 S, 14—23 S and 3—13 S, elute at 48, 63 and 69% of the bed volume, and account for 22, 13 and 62%, respectively, of the total protein eluted.

Interaction of heat-aggregated immunoglobulins with human leukocytes. The capacity of the various immunoglobulin fractions to engage cell surface Fc receptors was judged on the basis of lysosomal enzyme (β-glucuronidase) secretion. It is well established that the phagocytes of man and other species selectively release lysosomal, but not cytoplasmic, constituents when they are exposed to particulate challenges such as zymosan or immune complexes [23]. In the absence of phagocytic stimuli, cells secreted only 1.1% of their total lysosomal β-glucuronidase and cytoplasmic lactate dehydrogenase during a 15 min incubation (Table III). Quite dramatically, cells were stimulated to release β-glucuronidase (Table III) by immunoglobulin G fractions in decreasing order: I > II > III ⩾ unfractionated immunoglobulin G. Thus, the effectiveness of the various fractions to stimulate lysosomal enzyme secretion appeared to be a function of the size of the aggregates. The ability of serum-treated zymosan (C3b-coated), which presumably interacts with cells via C3-receptors, to stimulate enzyme release was tested as a positive control to show that the cells were capable of a response. Serum-treated zymosan induced a modest 6% release of β-glucuronidase. Co-release of the cytoplasmic enzyme lactate dehydrogenase was negligible at all times, ranging between 0.6 and 1.9% of total cellular activity, indicating that cell viability was not compromised by any of the particulate challenges and that the enzyme secretion was specifically lysosomal.

Ultrastructural analyses of liposome-cell interaction. Ultrastructural cytochemistry was performed to determine which cell type (in heterogeneous populations) was most active in the uptake of liposomes and whether liposomes were localized in the lysosomes or cytoplasm rather than adhering to the cell

TABLE III

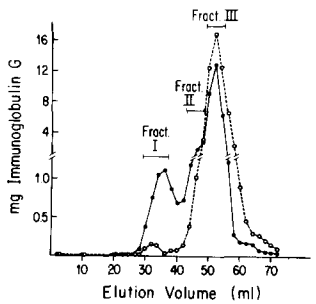
INDUCTION OF LYSOSOMAL ENZYME RELEASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES BY AGGREGATED IMMUNOGLOBULIN G FRACTIONS

Leukocytes, isolated either by the dextran-sedimentation method (n = 4) or the Hypaque/Ficoll gradient procedure (n = 3), were preincubated in the presence of cytochalasin B (5 μg/ml) for 10 min at 37°C; incubation of cells ((1—2) · 10^6/ml) with the respective stimuli was for 15 min at 37°C.

Treatment	β-Glucuronidase release (% of total ± S.E. (n = 7))
Control	1.1 ± 0.2
IgG fraction I (150 μg/ml)	14.3 ± 4.6
IgG fraction II (150 μg/ml)	10.3 ± 4.0 *
IgG fraction III (150 μg/ml)	6.1 ± 2.5 **
Unfractionated IgG (150 μg/ml)	2.7 ± 0.4 ***
Serum-treated zymosan (1 mg/ml)	5.9 ± 0.7

* vs. IgG fraction I, $P < 0.005$, Student's t-test.
** vs. IgG fraction II, $P < 0.05$, Student's t-test.
*** vs. IgG fraction III, not significant, Student's t-test.

surface. Human leukocytes, prepared by the dextran sedimentation method, were incubated with anionic multilamellar liposomes containing horseradish peroxidase [6]. The liposomes were either uncoated or coated with fraction I aggregates. In addition, a control incubation with empty liposomes was performed with an equivalent amount of non-encapsulated horseradish peroxidase. Cells washed free of the incubation medium were stained for ultrastructurally visible horseradish peroxidase by the Graham and Karnovsky technique [20]; the reaction product was visualized between the liposomal lamellae and distinguished liposomes from myelin figures (Fig. 4).



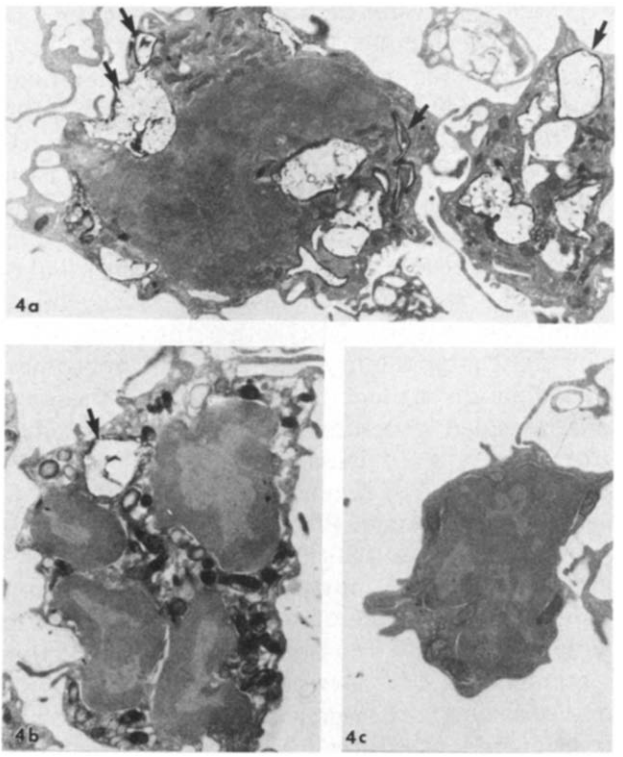


Fig. 3. Chromatographic fractionation of heat-aggregated human (•) and rabbit (○) immunoglobulin G using Bio-Gel A-5 M. Immunoglobulin G (60 mg) in 3.5 ml of Dulbecco's phosphate-buffered saline (17 mg protein/ml) was heated for 15 min at 62°C and then applied to a Bio-Gel A-5M (1.6 × 40 cm) column. Fractions were eluted as 2-ml aliquots with phosphate-buffered saline at a flow rate of 7.5 ml/h, and were analyzed for protein contents.

Fig. 4. Ultrastructure of leukocytes following 5 min incubation with liposomes coated with immunoglobulin G aggregates (fraction I), and containing horseradish peroxidase. a. A section through two adjacent monocytes showing multiple liposomes (associated with horseradish peroxidase reaction product) within the cytoplasm. The cell on the left was sectioned through the nucleus which excluded liposomes. Intracellular liposomes, a few of which are indicated by arrows, were present in both swollen phagocytic vacuoles and in compact vacuoles. Magnification, 8000X. b. A neutrophil showing one liposome-containing vacuole (arrow) and many myeloperoxidase-positive granules. Magnification, 10 000X. c. A lymphocyte from the same cell preparation as a and b, showing the complete absence of both liposomes and myeloperoxidase-positive granules. Magnification, 9350X.

Following 5 min incubation with aggregated IgG-coated liposomes, 83% of the monocytes (n = 41) in the 'mixed PMN' cell population had multiple liposome profiles whereas only 28% (n = 146) of the neutrophils were liposome-positive. Very few of the polymorphonuclear leukocytes contained more than one liposome. After 10 min incubation, 100% of the monocytes (n = 35) and 37% of the neutrophils (n = 130) contained liposomes, but the number of liposomes per neutrophil profile had not increased. When aggregated immunoglobulin-G-coated liposomes were incubated for 5 min with cells of the 'mononuclear fraction', it was observed that of 455 lymphocyte profiles (myeloperoxidase-negative granules) examined, none contained liposomes; in contrast, 85% of the monocytes (n = 68) (myeloperoxidase-positive granules) contained one or more multilamellar liposomes.

There are several limitations in quantifying liposome uptake using this ultrastructural technique. Liposomes containing little horseradish peroxidase, either because of their small size or because they had been ruptured, would be difficult to visualize cytochemically. Furthermore, the exchange of lipids between the liposomal and cellular membranes would be undetectable. Thus, ultrastructural cytochemistry will accurately reflect actual uptake only when the liposomes taken up contain enough peroxidase to be visualized in situ.

These experiments suggest that monocytes took up liposomes more actively than lymphocytes and polymorphonuclear leukocytes. Moreover, the liposomes associated with monocytes and neutrophils were overwhelmingly present within the cells (n = 352) rather than adherent to the outer plasma membrane (n = 1). The possibility that adherent liposomes are under-represented because of a fixation artifact is unlikely since the same fixation technique has previously yielded structurally intact liposomal membrane adsorbed to cell surfaces (for other types of cells).

Optimization of liposome uptake by coating liposomes with immunoglobulin aggregates. A 'mixed PMN' cell population, isolated by the dextran sedimentation method, was utilized to determine which fraction of aggregated immunoglobulin G would most enhance liposomal uptake. The uptake of entrapped inulin in the absence of serum increased significantly by 48% when liposomes were coated with fraction I (Table IV A); this increase in inulin uptake was matched by a 14% increase in the uptake of phospholipid. In contrast, coating of liposomes with aggregated immunoglobulin G fractions II, III, or with unfractionated aggregates did not enhance cellular internalization of either encapsulated inulin or phospholipid. In addition, uncoated liposomes incubated with cells, in the presence of serum, produced a 4.6-fold increase in the cellular association of encapsulated inulin as well as an 11% decrease in phospholipid entry (Table IV. A).

The ratio of cell-associated liposomal markers (ng inulin/nmol phospholipid) was 6 after serum-free incubation and 29 after incubation in the presence of serum, as compared to 55 ± 15 ($\bar{x}$ ± S.D.; n = 19) for the original liposomal suspension employed. The difference in the ratio of cell-associated markers (± serum) was roughly the same regardless of whether or not liposomes were immunoglobulin-G-coated. This observation is consistent with either of two alternatives. Firstly, the ratio may reflect the size distribution of the liposomes preferentially interacting with the cells. One would expect that the ratio of inulin

TABLE IV

LIPOSOME UPTAKE BY THREE POPULATIONS OF HUMAN LEUKOCYTES AS A FUNCTION OF IMMUNOGLOBULIN G COATING

Three different populations of normal human leukocytes were isolated and incubated with liposomes, either in the presence or absence of serum. Liposomes were labelled with ^{3}H-labelled phosphatidylcholine and [^{14}C]inulin serving as lipid phase and aqueous phase markers, respectively; they were either uncoated or coated with various aggregated human IgG fractions (100 μg/ml). The total amounts of liposomal lipid and liposome-entrapped inulin incubated per 10^6 cells equalled 330 ± 140 nmol lipid and 16 400 ± 9200 ng inulin ($\bar{X}$ ± S.D., n = 13), respectively. Cell association of liposomal markers was determined after 15 min of incubation at 37°C and is expressed as mean value ± S.E. (n). 'Mixed' PMN is comprised of monocytes (3 ± 2%), lymphocytes (12 ± 3%), PMN leukocytes (78 ± 4%), eosinophils (6 ± 2%) and basophils (0.9 ± 0.8%); $\bar{X}$ ± S.D., n = 5. 'Pure' PMN is comprised of monocytes (1.1 ± 0.6%), lymphocytes (3 ± 2%), PMN leukocytes (88 ± 3%), eosinophils (8 ± 2%) and basophils (0.4 ± 0.5%); $\bar{X}$ ± S.D., n = 5. 'Mononuclear' is comprised of monocytes (30 ± 6%), lymphocytes (61 ± 7%) and PMN leukocytes (9 ± 5%), $\bar{X}$ ± S.D., n = 5.

Cell population	Liposome coating	Liposomal marker uptake					
		In the absence of serum		Ratio, inulin : lipid (ng/nmol)	In the presence of serum		Ratio, inulin : lipid (ng/nmol)
		(nmol lipid/10^6 cells per 15 min)	(ng inulin/10^6 cells per 15 min)		(nmol lipid/10^6 cells per 15 min)	(ng inulin/10^6 cells per 15 min)	
A. Mixed PMN	None	5.7 ± 0.1 (36)	32 ± 4 (36)	6	5.1 ± 1.1 (4)	146 ± 10 (4)	29
	Fraction I	6.5 ± 0.3 (7) *	47 ± 10 (7) *	7	3.0 ± 1.0 (4)	80 ± 24 (4) *	27
	Fraction II	6.0 ± 0.6 (4)	30 ± 13 (4)	5	4.3 ± 1.2 (4)	117 ± 17 (4)	27
	Fraction III	5.9 ± 0.3 (4)	33 ± 12 (4)	6	4.3 ± 1.0 (4)	123 ± 4 (4) *	29
	Unfractionated IgG	5.9 ± 0.4 (7)	36 ± 9 (7)	6	3.4 ± 0.9 (4)	92 ± 12 (4) *	27
B. Pure PMN	None	2.3 ± 0.1 (37)	16 ± 1 (37)	7	1.6 ± 0.4 (4)	68 ± 6 (4)	43
	Fraction I	3.6 ± 0.2 (9) *	17 ± 3 (9)	5	1.5 ± 0.6 (4)	28 ± 16 (4) *	19
C. Mononuclear	None	3.2 ± 0.8 (4)	65 ± 12 (4)	20	1.6 ± 0.4 (3)	126 ± 24 (3)	79
	Fraction I	3.5 ± 0.7 (4) **	80 ± 16 (4) **	22	0.9 ± 0.3 (3)	71 ± 27 (3)	79

* vs. 'uncoated' control; $P < 0.05$, Student's t-test for two independent means.

** vs. 'uncoated' control; $P < 0.05$, Student's t-test for paired observations.

to phospholipid would be proportional to the size of the liposomes taken up, with the smallest liposomes in the heterogeneously sized population having the lowest entrapment volume per nmol lipid. Secondly, the difference in ratio of markers may reflect a difference in the mode of liposomal entry. A low ratio would be observed if the liposomes leaked prior to uptake by cells or if phospholipid exchange was a major component of the interactive process. The second hypothesis is less likely than the first since: (i) leakage of inulin from liposomes, as mediated by serum [4], would be expected to decrease the ratio (inulin : lipid) of cell-associated liposomal markers in the presence of serum, in contrast to the results obtained, and (ii) enhanced exchange of phospholipid would be expected to decrease the ratio (inulin : lipid) by increasing the lipid uptake, in contrast to the results obtained.

In order to confirm our ultrastructural observation that monocytes, rather than polymorphonuclear leukocytes or lymphocytes, are primarily responsible for liposome uptake, we performed a parallel series of experiments with a 'pure PMN' cell population isolated by the Hypaque/Ficoll gradient procedure (Table IV. B). This cell preparation was more homogeneous, and characteristically consisted of 88% polymorphonuclear leukocytes, 1.1% monocytes, 3% lymphocytes, 8% eosinophils and 0.4% basophils. It was our expectation that a reduction in the relative number of monocytes in the population would cause a decrease in the degree of uptake of coated and uncoated liposomes. In fact, we did observe a 50% reduction in the uptake of encapsulated inulin, both in the presence and absence of serum, as well as 60—70% reduction in the uptake of phospholipid marker. Although relatively less active in liposome uptake, the cells of this population responded to the presence of serum: 4.3-fold increases were found in uptake of encapsulated inulin. One should note that the ratio of the cell-associated markers (inulin : phospholipid) was several-fold greater when the incubation was performed in the presence of serum: this was consistent with our experience using the 'mixed PMN' cell population. In the absence of serum, coating of liposomes with aggregated immunoglobulins of fraction I resulted in a 56% enhancement of phospholipid uptake but did not influence encapsulated inulin uptake.

In a third series of experiments, we employed a predominantly 'mononuclear' cell fraction (isolated from Hypaque/Ficoll gradients) which was composed of 30% monocytes, 61% lymphocytes, and 9% polymorphonuclear leukocytes (Table IV C). In the absence of serum, these cells internalized about 2-fold more encapsulated inulin (coated and uncoated liposomes) than the 'mixed PMN' population cells, and 4-fold more than the 'pure PMN' population cells. Both lipid and inulin uptake by the cells (in the absence of serum) was enhanced significantly when liposomes were coated with aggregated immunoglobulin G fraction I (Table IV C). Liposomes coated with fraction I of human immunoglobulin G at a concentration of 100 μg/ml (16 μg/μmol lipid) proved to be the most effective inulin carrier (Table V), significantly enhancing inulin marker uptake by 63% over that induced by uncoated (control) liposomes. This concentration of immunoglobulin G closely corresponds to the amount of protein (rabbit) that chromatographically co-eluted with the liposome peak in the binding experiment. As seen for the other cell populations, the mononuclear cells internalized more inulin from uncoated liposomes when serum was present.

We also examined the consequences of preincubating the 'mononuclear' cells without serum at 37°C for 45 min prior to the addition of immunoglobulin-G-coated liposomes (Table VI). The uptake of immunoglobulin-G-coated liposomal inulin was increased by 2-fold with this preincubation, while uncoated liposomal association with cells was not altered significantly. The ratio of liposomal (immunoglobulin-G-coated) inulin to phospholipid becoming associated with cells increases after 45 min of preincubation, suggesting a preferential internalization of larger vesicles from the heterogeneously sized population. These effects may be due to the release of previously bound ligands from cell surface receptors during the preincubation period, thereby allowing the subsequent interaction of the immunoglobulin G aggregates (fraction I) coating the liposomes with vacant Fc receptors.

Since it was conceivable that the cellular entry of inulin may proceed independently of liposomal carriers following leakage from liposomes, we evaluated the potential contribution of pinocytosis to the overall uptake process. Aliquots of the three different populations of cells were incubated for 15 min with a quantity of inulin equaling that presented within liposomes (Table IV). Pinocytosis of free inulin by the 'mixed PMN', 'pure PMN', and 'mononuclear' cell populations amounts of 5 ± 2 (n = 13), 1 ± 2 (n = 3), and 3 ± 1 (n = 4) ng inulin/10^6 cells per 15 min ($\bar{X}$ ± S.D.; (n)), respectively, regardless of whether or not serum or empty liposomes were present. Therefore, even if the liposomes were fully disrupted and leaked all their cargo of inulin into the medium, pinocytosis could contribute only 6—15% of the uptake observed in the absence of serum and 1—3% of the uptake observed in the presence of serum. It is conceivable that pinocytosis could become a major factor in total observed uptake if large numbers of liposomes adsorbed to the cell surfaces and released inulin locally; however, this is unlikely since very little adherence of liposomes to cells was observed using ultrastructural techniques.

TABLE V

OPTIMIZATION OF LIPOSOME UPTAKE BY MONONUCLEAR CELLS AS A FUNCTION OF IMMUNOGLOBULIN CONCENTRATION DURING COATING

Multilamellar liposomes, doubly labelled with ^{3}H-labelled phosphatidylcholine (lipid phase) and [^{14}C]-inulin (aqueous phase), were coated with human immunoglobulin G fraction I aggregates at various concentrations. After 1 h incubation at 37°C of liposomes with mononuclear cells, the association of liposomal markers with cells was determined. The 'control' value for the uptake of uncoated liposomal phospholipid was 5.1 ± 0.6 nmol lipid/10^6 cells per 60 min ($\bar{X}$ ± S.E.; n = 8). The 'control' value for the uptake of uncoated liposome-entrapped inulin was 144 ± 23 ng inulin/10^6 cells per 60 min ($\bar{X}$ ± S.E.; n = 8). Values represent % of control ± S.E. (n).

IgG concentration during coating of liposomes (μg/ml)	Liposomal uptake	
	Phospholipid	Inulin
0	100 (8)	100 (8)
50	97 ± 7 (6)	145 ± 29 (6)
100	99 ± 9 (8)	163 ± 16 * (8)
200	85 ± 8 (5)	146 ± 24 (5)
300	87 ± 11 (2)	138 ± 6 (2)

* vs. control; $P < 0.005$, one sample Student's t-test statistics for the mean.

TABLE VI

EFFECTS OF PREINCUBATION OF MONONUCLEAR CELLS ON LIPOSOME UPTAKE

Mononuclear cells were preincubated at 37°C in serum-free phosphate-buffered saline for either 0 or 45 min prior to the introduction of a liposomal suspension. The doubly radioactively labelled liposomes were either uncoated or coated with aggregated human immunoglobulin G fraction I at 100 μg/ml.

Preincubation (min)	IgG-coated liposomes		Ratio, inulin : lipid (ng/nmol)	Uncoated liposomes		Ratio, inulin : lipid (ng/nmol)
	(nmol lipid/10^6 cells per 15 min)	(ng inulin/10^6 cells per 15 min)		(nmol lipid/10^6 cells per 15 min)	(ng inulin/10^6 cells per 15 min)	
0	3.5 ± 0.7 (4)	80 ± 16 (4)	23	3.2 ± 0.8 (4)	65 ± 12 (4)	20
45	3.3 ± 0.5 (5)	175 ± 32 (5) *	53	2.9 ± 0.5 (5)	90 ± 17 (5)	31

* vs. control (0 min preincubation); $P < 0.025$, Student's t-test for two independent means.

Discussion

The interactions of liposomes with phagocytic leukocytes have been studied previously in several cellular systems: blood phagocytes of smooth dogfish [6], mixed blood leukocytes (dextran-sedimented) from Tay-Sachs patients [7], rabbit peritoneal polymorphonuclear leukocytes [24,25], and mouse peritoneal macrophages [26—28]. The uptake of liposomes by cells was suggested to occur via either phagocytosis [6,7,27] when liposomes were precoated with aggregated immunoglobulins, or fusion when liposomes were uncoated [24,25].

All these studies [6,7,24—28] have a number of limitations. Several investigations relied on the uptake of biological activities (hexosaminidase A, Ref. 6; horseradish peroxidase, Ref. 7; macrophage activating factor, Ref. 28) by recipient cells or on the fate of lipid phase markers alone [24,25,27] and failed to employ multiple markers for the aqueous compartment and the lipid phase. Even though encapsulated enzymes were shown to be catalytically latent, it was possible that the 'entrapped' protein molecules were partially exposed on the liposome surface and provided a suitable surface ligand for particular cell types, thereby producing an atypical estimation of liposome-cell interaction; indeed, this possibility is supported by the demonstration in vivo that β-glucuronidase-loaded liposomes are more rapidly accumulated in liver than empty liposomes [29]. Investigations relying entirely on ultrastructural analyses are subject to sampling error [26].

In the present study, the kinetics of cellular association of liposomal radioactively labelled markers (aqueous phase and lipid phase) were correlated with an ultrastructural analysis of cells interacting with horseradish-peroxidase-containing liposomes. These data demonstrated that the uptake of liposomes by normal human leukocytes was linear for 10—15 min and was mediated by a saturable, energy-dependent, surface-dependent process. Ultrastructural cytochemistry revealed that monocytes were several-fold more actively engaged in the uptake of liposomes than were polymorphonuclear leukocytes and lymphocytes, on a per cell basis; this observation was confirmed by comparing the degree of uptake of radiolabelled liposomes by a 'pure PMN' population, a 'mixed PMN' population, and a 'mononuclear cell' population.

The introduction of liposomes into human leukocytes could be maximized either in the presence of serum or by precoating the liposomes with a high molecular weight fraction of heat-aggregated immunoglobulin G. However, when cells in the presence of serum were incubated with liposomes coated with immunoglobulin G fraction I aggregates, their uptake of liposomal inulin was reduced by 45—60%. These observations are consistent with the concept that serum factors (complement components, fibronectin, immunoglobulins, etc.) bind to liposomes and expedite their interaction with specific cell surface receptors. By coating the liposomes with fraction I aggregates, we can imitate the enhancement effect of serum. The diminution of liposomal inulin entry into cells that was observed in the presence of serum as a function of coating the liposomes with immunoglobulin G fraction I suggests that either (i) the immunoglobulin G aggregates bound to the liposomes partially obstruct subsequent liposomal membrane interaction with serum factors or (ii) free immunoglobulin G aggregates (in equilibrium with liposome-bound molecules) may bind to cell surface Fc receptors and partially block subsequent interaction with serum factor-coated liposomes. This latter explanation is both in

accord with our observation that liposomes coated with aggregates of immunoglobulin G (fraction I) are internalized by monocytes to a greater extent when the monocytes are preincubated in serum-free medium and with our finding that the presence of excess numbers of free immunoglobulin G aggregates block the uptake of liposomes by leukocytes. This interpretation is also supported by the recent work of Leserman et al. [30], who have demonstrated the Fc receptor-mediated endocytosis of immunoglobulin-G-opsonized liposomes by phagocytic murine tumor cells.

Acknowledgments

This work was aided by grants from the National Science Foundation (PCM 78-13221), the American Lung Association, and New York University Medical Center (Biomedical Research Support Grant) to Morris Finkelstein, and grants (AM-11949, HL-19721) from the National Institutes of Health and the Arthritis Foundation to Gerald Weissmann.

References

1 Weissmann, G., Korchak, H., Finkelstein, M., Smolen, J. and Hoffstein, S. (1978) Ann. N.Y. Acad. Sci. 308, 235—247
2 Finkelstein, M. and Weissmann, G. (1978) J. Lipid Res. 19, 289—303
3 Finkelstein, M.C., Maniscalco, J. and Weissmann, G. (1978) Anal. Biochem. 89, 400—407
4 Finkelstein, M.C. and Weissmann, G. (1979) Biochim. Biophys. Acta 587, 202—216
5 Sessa, G. and Weissmann, G. (1970) J. Biol. Chem. 245, 3295—3301
6 Weissmann, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gottlieb, A. and Nagle, D. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 88—92
7 Cohen, C.M., Weissmann, G., Hoffstein, S., Awasthi, Y.C. and Srivastava, S.K. (1976) Biochemistry 15, 452—460
8 Weissmann, G., Cohen, C. and Hoffstein, S. (1977) Biochim. Biophys. Acta 498, 375—385
9 Magee, W.E., Goff, C.W., Schoknecht, J., Smith, M.D. and Cherian, K. (1974) J. Cell Biol. 63, 492—504
10 Sandra, A. and Pagano, R.E. (1979) J. Biol. Chem. 254, 2244—2249
11 Weissmann, G., Brand, A. and Franklin, E.C. (1974) J. Clin. Invest. 53, 536—543
12 Schieren, H., Weissmann, G., Seligman, M. and Coleman, P. (1978) Biochem. Biophys. Res. Commun. 82, 1160—1167
13 Schieren, H. (1980) Ph.D. Dissertation, New York University, New York
14 Rudolf, S. (1980) Ph.D. Dissertation, New York University, New York
15 Goldstein, I., Hoffstein, S., Gallin, J. and Weissmann, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2916—2920
16 Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97), 77—89
17 Finkelstein, M.C. and Adelberg, E.A. (1977) J. Biol. Chem. 252, 7101—7108
18 Schieren, H., Rudolf, S., Finkelstein, M., Coleman, P. and Weissmann, G. (1978) Biochim. Biophys. Acta 542, 137—153
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Graham, R.C., Jr. and Karnovsky, M.J. (1966) J. Histochem. Cytochem. 14, 291—302
21 Smolen, J.E. and Weissmann, G. (1978) Biochim. Biophys. Acta 512, 525—538
22 Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. (1977) Ann. Rev. Biochem. 46, 669—722
23 Unanue, E.G. (1976) Am. J. Pathol. 83, 396—418
24 Stendahl, O. and Tagesson, C. (1977) Exp. Cell. Res. 108, 167—174
25 Dahlgren, C., Kihlstrom, E., Magnusson, K.E., Stendahl, O. and Tagesson, C. (1977) Exp. Cell Res. 108, 175—184
26 Wiktorowicz, J.E., Baur, P.S. and Srivastava, S.K. (1977) Eur. J. Cell. Biol. 14, 401—411
27 Mattenberger-Kreber, L., Auderset, G., Schneider, M., Louis-Broillet, A., Strolin-Benedetti, M. and Malnoe, A. (1976) Experientia 32, 1522—1524
28 Poste, G., Kirsh, R., Fogler, W.E. and Fidler, I.J. (1979) Cancer. Res. 39, 881—892
29 Steger, L.D. and Desnick, R.J. (1977) Biochim. Biophys. Acta 464, 530—546
30 Leserman, L.D., Weinstein, J.N., Blumenthal, R. and Terry, W.D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4089—4093